PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 03 August 2000 (03.08.00)	
International application No. PCT/EP99/10179	**Applicant's or agent's file reference** T/98421 WO
International filing date (day/month/year) 16 December 1999 (16.12.99)	**Priority date** (day/month/year) 18 December 1998 (18.12.98)
Applicant YAP, Sing, Hiem et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

24 June 2000 (24.06.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Hermans, Franciscus G.M.
P.O. Box 20
NL-5340 BH Oss
PAYS-BAS

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

	Date of mailing *(day/month/year)* 12.03.2001
Applicant's or agent's file reference T/98421 WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/10179	16/12/1999	18/12/1998

Applicant
AKZO NOBEL N.V. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Guerin, A Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
T/98421 WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/10179	16/12/1999	18/12/1998
International Patent Classification (IPC) or national classification and IPC C12N15/51		
Applicant AKZO NOBEL N.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
24/06/2000	12.03.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Lanzrein, M Telephone No. +49 89 2399 7358

Form PCT/IPEA/409 (cover sheet) (January 1994)

I. Basis of the r port

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-96 as originally filed

Claims, No.:

1-14 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

Sequence listing part of the description, pages:

1-11, filed with the letter of 13.04.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 4-7, 10-14.

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 4-7, 10-14 are so unclear that no meaningful opinion could be formed (*specify*):
   see separate sheet

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1, 3, 8, 9
	No:	Claims 2
Inventive step (IS)	Yes:	Claims 1, 3, 8, 9
	No:	Claims 2
Industrial applicability (IA)	Yes:	Claims 1-3, 8, 9
	No:	Claims

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Reference is made to the following documents:

D1: NISHIZAWA T ET AL: 'A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology' BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 241, no. 1, 8 December 1997 (1997-12-08), pages 92-97, cited in the application

D2: Fourneau I. et al.: Development and characterization of immortalized human hepatocyte lines and the application in a bioartificial liver device. In: Bioartificial liver support systems, the critical issues. Crapaldia G, Demetriou AA, Muraca M (Eds). Pages 62-69. 1997.
Cited by the applicant.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 4, 5, 6 and 12 refer to a "genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO:1". Since the sequence of said "genome" is unknown, the subject-matter of said claims is completely unclear and not searchable. It comprises any unknown sequence or polypeptide present or encoded by said undefined "genome". Therefore, a meaningful examination with regard to novelty and inventive step of said claims is at present not possible.
Similarly, examination of the subject-matter of appending claims 7, 10, 11, 13, 14 is at present not possible.

Re Item IV

Lack of unity of invention

The international preliminary examining Authority is of the opinion that the present application lacks unity within the meaning of Art. 34(3) and Rule 13.1 PCT.

It will be considered that the following separate alleged inventions are not so linked as to form a single general inventive concept:

1) Claim 1 (completely): A method for detecting non-A-G hepatitis virus.

2) Claim 2 (completely), 3 (partially): A human hepatocyte cell line (ECACC 98121503).

3) Claims 4-14 (completely) : Hepatitis Y virus, related nucleic acid sequences, polypeptides, antibodies, methods for detection and diagnosis, method for growing the virus, vaccines.

The general inventive concept underlying the alleged inventions 1) and 3) can be seen in the identification and detection of non-A-G hepatitis viruses. However, D1 describes the identification of TTV, another non-A-G virus. Thus, in view of D1, the inventive concept is not novel.
Independent claim 2 solely refers to a cell line, without reference to its use or technical features. Hence, due to the present wording of claim 2, no special features are apparent which are common between alleged invention 2) and alleged inventions 1) or 3).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Claim 2 lacks novelty according to Art. 33 (2) PCT over D2. This document discloses the establishment and characterization of several hepatocyte cell lines. The cell line C therein appears to be identical to the cell line of claim 2 which is described in more detail in the description p. 63-65. The values given in table 6 for cytokeratin expression, bilirubin conjugation and protein secretion are identical to the values given for cell line C in D2. It must thus be assumed that the two cell lines are identical and therefore, D2 is considered prejudicial to novelty of claim 2.

2. Claims 5 and 6 (and appending claims 7, 10, 11, 13, 14) lack novelty within the meaning of Art. 33 (2). The subject-matter is not being examined with regard to novelty (see item III). However, we would like to point out that said claims cannot be novel whatever the underlying subject-matter may be, because said claims are extending unlimitedly to fragments which may include trinucleotides (codons) or single amino acids.

3. Concerning claims 1, 3, 8, 9 the currently available prior art does not affect novelty or inventive step

Re Item VII

Certain defects in the international application

It appears as if the dependencies and references of the claims are wrong and unclear throughout the application:

1. Claims 7, 10 13 refer to a polypeptide of claim 3. However, claim 3 concerns the use of a cell line.

2. Claim 8, 14 refer to the nucleic acid of claim 2 which concerns a cell line.

3. Claim 9 refers to a sample of claim 5, but claim 5 concerns a nucleic acid sequence.

4. Claim 10 refers to a hepatitis Y virus according to claim 1. However, claim 1 concerns non-A-G hepatitis viruses.

5. Claim 11 refers to an antibody of claim 4 which, however, concerns hepatitis Y virus.

Re Item VIII

Certain observations on the international application

Claims 4-14 do not meet the requirements of Article 6 PCT

Claims 5, 6, 7 refer to "functional equivalents" of a nucleic acid sequence. This is totally unclear because neither the sequence nor any of the functions of putative proteins encoded by said sequence are disclosed.

Claims 5, 6 (and appending claims 7, 10, 11, 13, 14) refer to "fragments" of nucleic acids or polypeptides and no limitation to the size of the fragments is given. Therefore, the scope of protection is indefinite and the matter for which protection is sought is not clearly defined as required by Art. 6 PCT. Furthermore, since the scope extends to virtually to single nucleotides or amino acids, existing prior art molecules are prejudicial to novelty within the meaning of Art. 33 (2) PCT (see item V, 2.).

Claims 4, 5, 6, 12 refer to sequences "hybridisable" to a certain sequence. This is not clear under Art. 6 PCT because the subject-matter for which protection is sought is not precisely defined. Since no hybridisation conditions are included in the claims, they are to be understood as extending to any known prior art nucleic acid molecule, which would also deprive the claims of novelty in the sense of Art. 33 (2) PCT.
Similarly, claim 8 does not recite the conditions under which the nucleic acids hybridise.

The name "hepatitis Y virus" used in claims 4, 5, 8-12 appears to be an arbitrary

denomination which is, at present, meaningless to the skilled person.
Furthermore, the applicants have not provided any clear-cut evidence showing that the identified sequences (SEQ ID NOs: 1 and 2) do in fact belong to a new viral agent. The applicants show that said sequences are associated with a transmittable agent and they are present in dense fractions on a CsCl gradient. However, these data cannot rule out that the alleged new virus represents a strain or a variant of any of the known hepatitis viruses. It may also be identical with a newly discovered virus (TTV), of which the entire genomic sequence has not yet been determined.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use

International Application No.

Ces pièces constituent la confirmation d'une demande déposée par la télécopie (téléfax).

International Filing Date

Dat de l'envoi de la télécopie (téléfax) : 16/12/9;

Name of receiving Office and "PCT Internatior

Nom de l'autorité auprès de laquell la télécopie (téléfax) a été déposée : EPO - Rijswijk

Applicant's or agent's file reference *(if desired) (12 characters maximum)* ***T/98421 WO***

Box No. I TITLE OF INVENTION

"Hepatitis Y Virus"

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below)*

Akzo Nobel N.V.
Velperweg 76
6824 BM ARNHEM
The Netherlands

☐ This person is also inventor

Telephone No.
0412-666380

Facsimile No.
0412-650592

Teleprinter No.

State *(i.e. country)* of nationality: *The Netherlands*

State *(i.e. country)* of residence: *The Netherlands*

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below)*

YAP, S.H.
Sing Hiem
Kraaikant 22 A
3221 Nieuwrode (Holsbeek)
Belgium

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: *The Netherlands*

State *(i.e. country)* of residence: *Belgium*

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.*

F.G.M. Hermans
P.O. Box 20
5340 BH Oss
The Netherlands

Telephone No.
0412-666223

Facsimile No.
0412-650592

Teleprinter No.

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box N . III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below)*

VERSLYPE, C.J.C.
Chris Julien Cornelius
Dietsesteenweg 644
3010 Kessel-Lo
Belgium

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: *Belgium*

State *(i.e. country)* of residence: *Belgium*

This person is applicant for the purposes of
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below)*

BOENDER, P.
Piet
Ubbergseweg 58
6522 KJ Nijmegen
The Netherlands

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: *The Netherlands*

State *(i.e. country)* of residence: *The Netherlands*

This person is applicant for the purposes of
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below)*

HELLINGS, J.A.
Jan Albert
Lierenbout 24
5283 AT Boxtel
The Netherlands

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: *The Netherlands*

State *(i.e. country)* of residence: *The Netherlands*

This person is applicant for the purposes of
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- [x] **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [x] **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [x] **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [x] **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [x] **AL** Albania .....
- [ ] **AM** Armenia .....
- [ ] **AT** Austria .....
- [x] **AU** Australia .....
- [ ] **AZ** Azerbaijan
- [x] **BA** Bosnia and Herzegovina .....
- [x] **BB** Barbados
- [x] **BG** Bulgaria .....
- [x] **BR** Brazil .....
- [ ] **BY** Belarus .....
- [x] **CA** Canada
- [ ] **CH and LI** Switzerland and Liechtenstein
- [x] **CN** China .....
- [x] **CU** Cuba .....
- [x] **CZ** Czech Republic .....
- [ ] **DE** Germany .....
- [ ] **DK** Denmark .....
- [x] **EE** Estonia .....
- [ ] **ES** Spain .....
- [ ] **FI** Finland .....
- [ ] **GB** United Kingdom
- [ ] **GD** Grenada
- [x] **GE** Georgia .....
- [ ] **GH** Ghana .....
- [ ] **GM** Gambia
- [ ] **HR** Croatia .....
- [x] **HU** Hungary .....
- [x] **ID** Indonesia
- [x] **IL** Israel .....
- [x] **IN** India .....
- [x] **IS** Iceland
- [x] **JP** Japan .....
- [ ] **KE** Kenya .....
- [ ] **KG** Kyrgyzstan .....
- [x] **KP** Democratic People's Republic of Korea .....
- [x] **KR** Republic of Korea .....
- [ ] **KZ** Kazakhstan .....
- [x] **LC** Saint Lucia
- [x] **LK** Sri Lanka
- [x] **LR** Liberia
- [ ] **LS** Lesotho .....
- [x] **LT** Lithuania
- [ ] **LU** Luxembourg
- [x] **LV** Latvia
- [ ] **MD** Republic of Moldova .....
- [x] **MG** Madagascar .....
- [x] **MK** The former Yugoslav Republic of Macedonia .....
- [x] **MN** Mongolia
- [ ] **MW** Malawi .....
- [x] **MX** Mexico .....
- [x] **NO** Norway
- [x] **NZ** New Zealand .....
- [x] **PL** Poland .....
- [ ] **PT** Portugal .....
- [x] **RO** Romania
- [x] **RU** Russian Federation .....
- [ ] **SD** Sudan
- [ ] **SE** Sweden
- [x] **SG** Singapore
- [x] **SI** Slovenia .....
- [x] **SK** Slovakia .....
- [x] **SL** Sierra Leone
- [ ] **TJ** Tajikistan .....
- [ ] **TM** Turkmenistan .....
- [x] **TR** Turkey .....
- [x] **TT** Trinidad and Tobago .....
- [x] **UA** Ukraine .....
- [ ] **UG** Uganda .....
- [x] **US** United States of America .....
- [x] **UZ** Uzbekistan .....
- [x] **VN** Viet Nam .....
- [x] **YU** Yugoslavia .....
- [ ] **ZW** Zimbabwe .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [x] ZA South Africa .....
- [ ] .....
- [ ] .....

Precauti nary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration f 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) *NL*	*18-12-1998*	*98204313.5*	*EPO*
item (2) *NL*	*20-01-1999*	*99200167.7*	*EPO*
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

[X] The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s) : *1 and 2*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA */EPO*

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request*

Country (or regional Office): *EP* Date *(day/month/year)* *18-12-1998* Number: *98204313.5*

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	*4*	sheets
2. description:	*96*	sheets
3. claims:	*2*	sheets
4. abstract:	*1*	sheets
5. drawings	*6*	sheets
6.seq.listing	*16*	sheets
Total :	*125*	sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. [X] copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No VI* *as item(s):*
5. [X] fee calculation sheet
6. [X] separate indications concerning deposited microorganisms **(will follow)**
7. [X] nucleotide and/or amino acid sequence listing (diskette)
8. [X] other *(specify):* *Request refund Letter / Copy European Search Report*

Figure No. of the drawings ~~(if any)~~ should accompany the abstract when ~~it is~~ published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)

F.G.M. Hermans

For receiving Office use only

1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority specified by the applicant: ISA/	6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference *T/98421 WO*

Applicant
Akzo Nobel N.V.

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE ..... *Eur 102,=* | T

2. SEARCH FEE ..... *Eur 1124,=* | S

International search to be carried out by *EPO*
(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search .)

3. INTERNATIONAL FEE

Basic Fee
The international application contains *125* sheets.
first 30 sheets ..... *Eur 413,=* | b_1

95 remaining sheets X *10* additional amount = *950,=* | b_2

Add amounts entered at b_1 and b_2 and enter total at B ..... *Eur 1363,=* | B

Designation Fees
The international application contains *50* designations

number of designation fees payable *(maximun 10)* — amount of designation fee *Eur 950,=* | D

Add amounts entered at B and D and enter total at I ..... *Eur 2313,=* | I
(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT ..... *Eur 60,=* | P

5. TOTAL FEES PAYABLE
Add amounts entered at T, S, I and P, and enter total in the TOTAL box ..... *Eur. 3599,=*
TOTAL

☐ The designation fee is not paid at this time.

MODE OF PAYMENT

X	authorization to charge deposit account (see below)	☐	bank draft	☐	coupons
☐	cheque	☐	cash	☐	other *(specify):*
☐	postal money order	☐	revenue stamps		

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ *EPO*

X is hereby authorized to charge the total fees indicated above to my deposit account.

X is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized t charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

2809 0012 — Deposit Account Number

16-12-1999 — Date *(day/month/year)*

F.G.M. Hermans — Signature

See Notes to the fee calculation sheet

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant in the line below:

IPEA/ _____

PCT

CHAPTER III

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference *T/98421 WO*
International application No. *PCT/EP99/10179*	International filing date *(day/month/year* *16-12-1999*	(Earliest) Priority date *(day/month/year)* *18-12-1998*
Title of invention *"Hepatitis Y Virus"*		

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Akzo Nobel N.V.
Velperweg 76
6824 BM ARNHEM
The Netherlands

Telephone No.: *0412-666380*

Facsimile No.: *0412-650592*

Teleprinter No.:

State *(i.e. country)* of nationality: *NL*

State *(i.e. country)* of residence: *NL*

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country.)*

LAP, Sing Hiem
Kraaikant 22 A
3221 Nieuwrode (Holsbeek)
Belgium

State *(i.e. country)* of nationality: *NL*

State *(i.e. country)* of residence: *BE*

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country.)*

VERSLYPE, Chris Julien Cornelius
Dietsesteenweg 644
3010 Kessel-Lo
Belgium

State *(i.e. country)* of nationality: *BE*

State *(i.e. country)* of residence: *BE*

[X] Further applicants are indicated on a continuation sheet.

Sheet No. 2.....

International application No.
PCT/EP[redacted]179

Continuation of Box No. II **APPLICANT(S)**

If none of the following sub-boxes is used, this sheet is not to be included in the demand

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

BOENDER, Piet
Ubbergseweg 58
6522 KJ Nijmegen
The Netherlands

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:
NL	*NL*

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HELLINGS, Jan Albert
Lierenbout 24
'3 AT Boxtel
The Netherlands

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:
NL	*NL*

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:

e and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(i.e. country)* of nationality:	State *(i.e. country)* of residence:

☐ Further applicants are indicated on another continuation sheet.

International application No.
PCT/EP99/10179

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

F.G.M. Hermans
P.O. Box 20
5340 BH Oss
The Netherlands

Telephone No.: *0412-666382*

Facsimile No.: *0412-650592*

Teleprinter No.:

[ ] Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV STATEMENT CONCERNING AMENDMENTS

The applicant wishes the international Preliminary Examining Authority*

(i) [X] to start the international preliminary examination on the basis of the international application as originally filed.

(ii) [ ] to take into account the amendments under Article 34 of

- [ ] the description (amendments attached).
- [ ] the claims (amendments attached).
- [ ] the drawings (amendments attached).

(iii) [ ] to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).

(iv) [ ] to disregard any amendments of the claims made under Article 19 and to consider them as reversed.

(v) [ ] to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

[X] The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)* except.....
.....
.....
.....

(If the applicant does not wish to elect certain eligible States, the name(s) or country code(s) of those States must be indicated above.)

Sheet No. .4

International application No.
PCT/EP99/10179

Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:

			For International Preliminary Examining Authority use only: received	not received
1. amendments under Article 34				
description	:	sheets	☐	☐
claims	:	sheets	☐	☐
drawings	:	sheets	☐	☐
2. letter accompanying amendments under Article 34	:	sheets	☐	☐
3. copy of amendments under Article 19	:	sheets	☐	☐
4. copy of statement under Article 19	:	sheets	☐	☐
5. other *(specify)*:	:	sheets	☐	☐

. demand is also accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☒ fee calculation sheet
5. ☐ other *(specify)*:

Box N . VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

F.G.M. Hermans

For International Preliminary Examining Authority use

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.

4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.

5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

See Notes to the demand form

PCT

CHAPTER II

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No. *PCT/EP99/10179*

Applicant's or agent's file reference *T/98421 WO*

For International Preliminary Examining Authority use only

Date stamp of the IPEA

Applicant *Akzo Nobel N.V.*

Calculation of prescribed fees

1. Preliminary examination fee ..... *Euro 1533,=* P

2 Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)* ..... *Euro 148,=* H

3. Total of prescribed fees
Add the amounts entered at P and H and enter total in the TOTAL box ..... *Euro 1681,=* TOTAL

Mode of Payment

- [X] authorization to charge deposit account with the IPEA (see below)
- [ ] cheque
- [ ] postal money order
- [ ] bank draft
- [ ] cash
- [ ] revenue stamps
- [ ] coupons
- [ ] other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA / *EPO*

- [X] is hereby authorized to charge the total fees indicated above to my deposit account.
- [X] *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

2809 0012	*19 June 2000*	*F.G.M. Hermans*
Deposit Account Number	Date *(day/month/year)*	Signature

See Notes to the fee calculation sheet

PATENT COOPERATION TREATY

12 MEI 2000

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
Hermans, Franciscus G.M.
Attn. Hermans, Franciscus G.M
P.O. Box 20
NL-5340 BH Oss
NETHERLANDS

Applicant's or agent's file reference T/98421 WO	Date of mailing (day/month/year) 11/05/2000
	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/EP 99/ 10179	International filing date (day/month/year) 16/12/1999
Applicant AKZO NOBEL N.V. et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been lected in the demand or in a later election within 19 m nths from th priority date or could not be elected because th y are not bound by Chapter II.

Nam and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 po nl, Fax: (+31-70) 340-3016	Sandra De Jong-van Dam

Form PCT/ISA/220 (July 1998)

NOTE FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
T/98421 WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 99/ 10179	16/12/1999	18/12/1998
Applicant		
AKZO NOBEL N.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] th t xt is approved as submitted by th applicant.

   [ ] th text has been established, according to Rul 38.2(b). by this Authority as it appears in Box III. Th applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of th **drawings** to be published with th abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes th invention.

   [ ] Non of th figures.

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted] 99/10179

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/51 C07K14/08 C12Q1/68 G01N33/68 A61K39/29
C07K16/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KARAYIANNIS P ET AL: "STUDIES OF GB HEPATITIS AGENT IN TAMARINS" HEPATOLOGY, vol. 9, no. 2, 1 February 1989 (1989-02-01), pages 186-192, XP000561299 cited in the application the whole document	
A	LINNEN J ET AL: "MOLECULAR CLONING AND DISEASE ASSOCIATION OF HEPATITIS G VIRUS: A TRANSFUSION-TRANSMISSIBLE AGENT" SCIENCE, vol. 271, no. 3, 26 January 1996 (1996-01-26), pages 505-508, XP002028989 cited in the application the whole document	

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 May 2000	11/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MUERHOFF A S ET AL: "GENOMIC ORGANIZATION OF GB VIRUSES A AND B: TWO NEW MEMBERS OF THE FLAVIVIRIDAE ASSOCIATED WITH GB AGENT HEPATITIS" JOURNAL OF VIROLOGY, vol. 69, no. 9, 1 September 1995 (1995-09-01), pages 5621-5630, XP000612027 cited in the application the whole document	
A	NISHIZAWA T ET AL: "A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 241, no. 1, 8 December 1997 (1997-12-08), pages 92-97, XP002080817 cited in the application the whole document	
A	KUWADA S K ET AL: "NON-A, NON-B FULMINANT HEPATITIS IS ALSO NON-E AND NON-C" GASTROENTEROLOGY, vol. 89, no. 1, 1 January 1994 (1994-01-01), pages 57-61, XP000561262 cited in the application the whole document	
A	SING-HIEM YAP ET AL.: "Detection of hepatitis C virus antigen by immuno-histochemical staining: a histological marker of hepatitis C virus infection." JOURNAL OF HEPATOLOGY, vol. 20, no. 2, February 1994 (1994-02), pages 275-281, XP002103585 cited in the application the whole document	

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(54) Title: HEPATITIS Y VIRUS

(57) Abstract

The isolation and characterisation of a novel hepatitis virus is described. This virus was tentatively named Hepatitis Y Virus. The nucleic acid sequence of part of the genome is provided therewith enabling the detection of the novel virus in samples from patients suspected of suffering from infection with hepatitis Y virus. Also provided are polypeptide sequences and antibodies directed against them therewith enabling the detection of viral proteins in patient material. The invention also provides vaccines against Hepatitis Y Virus as well as methods for growing Hepatitis Y Virus in an *in vitro* cell line.

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HEPATITIS Y VIRUS

This invention is based on the discovery of a new form of human hepatitis, termed "Hepatitis Y". The invention relates to a viral agent associated with the disease, termed Hepatitis Y virus or HYV, and more in particular, relates to polynucleotide sequences derivable from this agent, polypeptides encoded by these polynucleotides, antibodies which specifically bind to these polypeptides and diagnostic methods and kits and vaccines that can be designed and made using these materials and the information that is embedded in these sequences. Moreover, the invention relates to an *in vitro* method for culturing HYV.

Hepatitis is one of the most important diseases transmitted from a donor to a recipient by transfusion of blood products, organ transplantation and hemodialysis; it also can be transmitted via ingestion of contaminated food and water, and by person-to-person contact. Viral hepatitis is known to be caused by a variety of viral agents with distinctive viral genes and modes of replication. Viral hepatitis is known to occur with differing degrees of severity of hepatic damage through different routes of transmission. In some cases, acute viral hepatitis can be clinically diagnosed by well-defined patient symptoms including jaundice, hepatic tenderness and an elevated level of liver transaminases such as aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH). In other cases, acute viral hepatitis may be clinically inapparent. The viral agents of hepatitis include hepatotropic viruses likeHepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV), and systemic viruses like Hepatitis E virus (HEV), Epstein-Barr virus (EBV) and Cytomegalovirus (CMV). The latter class of viruses is not associated with chronic hepatitis

HAV infection occurs world-wide and is an important cause of acute viral hepatitis. HAV was discovered in 1973 (Feinstone et al. Science 1973;182:1026-8) and is a positive sense, single-stranded RNA virus classified within the genus hepatovirus of the picornaviridae family. With genomic RNA, HAV can function as messenger in directing the translation of proteins. A single large polyprotein is expressed from a large open-reading frame that extends through most of the genomic RNA. Translation takes place in a cap-independent fashion under control of an internal ribosome entry segment located within the 5' untranslated RNA. The polyprotein subsequently undergoes cleavage mediated by a viral protease, resulting in the

production of four capsid proteins and several non-structural proteins. HAV, which lacks a lipid envelope, is stable when it is secreted from infected liver cells into the bile, and it gains entry to the intestinal tract via this route. Thus, this virus typically spreads by a faecal-oral mode of transmission, and can cause extensive common-source outbreaks of disease. In countries where the disease is endemic, most of the population are infected during childhood. There are few or no symptoms, and the infected individuals acquire life-long immunity to the virus infection. The development of antibodies to HAV coincides with a marked diminution in quantity of viremia and faecal shedding of the virus (Lemon et al, J. Inf. Dis 1983;148:1033-9; Lemon et al. J. Inf. Dis. 1990;161: 7-13). Detection of this acute-phase antibody response is the mainstay of diagnosis. Antibodies to HAV of the IgM class are present in more than 99% of patients at the time of their initial presentation. The IgM antibody appears during acute illness or shortly after infection and persists for several months; its detection is a marker of a present or a recent infection. Thereafter, antibodies of the IgG class appear and remain life-long, providing immunity to re-infection (Dienstag and Isselbacher, In: Isselbacher KJ, Braunwald E, Wilson JD, eds. Harrison's Principles of Internal Medicine, 13th Ed. New York: McGraw-Hill, 1994, pp 1458-78; Ramos-Soriano and Schetwartz, Gastroenterol. Clin. North Am. 1994;23:753-67). Recombinant and inactivated HAV vaccines are now available and have been proven to be both safe and effective (Katkov and Dienstag, Gastroenterol. Clin. North Am 1995; 24:147-59.)

HBV infection is world-wide, one of the most frequent viral infections in humans. HBV is a partially double-stranded circular DNA virus that belongs to the family of hepadnaviruses (or hepatotropic DNA viruses). The discovery of "Australia antigen" by Blumberg in 1964 (Blumberg, Bull. NY Acad. Med. 1964; 40:377) has finally led to the finding of Dane particle as the causative agent of this type of hepatitis. The Dane particle is the complete HB virion composed of a partially double-stranded DNA genome including the nucleocapsid (hepatitis B core antigen - HBcAg), which is coated by the hepatitis B surface antigen (HBsAg) (Dienstag and Isselbacher, 1994; Ramos-Soriano and Schwartz, 1994). The first serological marker which appears after HBV infection is HBsAg. This viral antigen can be detected from 2 to 12 weeks after infection with HBV. The presence of HBsAg often antedates symptoms or abnormalities of liver biochemistry by 6-8 weeks. In acute resolving disease, it becomes undetectable 1 to 2 months following the onset of jaundice and rarely persists beyond 6

months. In chronic HBV infection, HBsAg remains detectable beyond 6 months. In acute resolving disease, after HBsAg disappears, antibodies to HBsAg (anti-HBs) become detectable in serum and remain life-long providing immunity to re-infection. The IgM antibody to HBcAg (anti-HBc IgM) appears usually 2 weeks after the detection of HBsAg, and it remains detectable for up to 6 months after onset of acute hepatitis. Before the disappearance of this antibody, another class of antibody to HBcAg (anti-HBcAg IgG) appears and remains life-long. The detection of the anti-HBc IgM is of assistance in diagnosing an acute or recent infection in patients with HBsAg concentrations that are below the sensitivity threshold of the diagnostic assay. Another important serological marker of HBV infection, the HBAg (hepatitis B antigen), appears concurrently with or shortly after the appearance of HBsAg in serum and is a marker of viral replication. In addition to the serological markers, serum HBV-DNA has been found to be the most sensitive marker of viral replication it can be detected very early during the course of infection, especially if it is determined by an amplification technique (Dienstag et al., 1994; Ramos-Soriano and Schwartz, 1994).

In the early 1970s, when specific diagnostic assays for detection of HAV and HBV infections became available, it was clear that some cases of enterally and of parenterally transmitted hepatitis were not caused by these agents or by any other known systemic viruses (CMV, EBV, HSV etc.). Such cases were classified as non-A, non-B viral hepatitis.

The aetiology of non-A, non-B viral hepatitis was an enigma until 1989-1990 when it became more transparent by the cloning of the HCV genome (Choo et al., Science 1989;244:359-62) and the isolation of a cDNA clone of the HEV (Reyes et al., Science 1990 247: 1335-9). HCV is identified as the major cause of parentally transmitted non-A, non-B hepatitis (Alter HJ et al., N. Engl. J. Med. 1989; 321:1494-1500; Weiner et al.; Lancet 1990; 335: 1-3). At that time more than 50% of all cases of non-A, non-B chronic hepatitis was found to be related to HCV infection (Hammel et al., J. Hepatol.1994; 21: 618-23), while HEV was found as the main causative agent for enterally transmitted non-A, non-B viral hepatitis (Reyes et al., 1990; Bradley, Br. Med. Bull. 1990; 46:442-61; Velazquez, JAMA 1990; 263: 3281-5) especially in Asia and Africa

HCV, the major causative agent of post-transfusion non-A, non-B hepatitis, poses a serious world-wide health problem. HCV is a spherical enveloped virus of approximately 50

nm in diameter (Shimizu et al, 1996). The genome of HCV is a positive single-stranded RNA virus (Choo et al., 1989; Dienstag and Isselbacher, 1994; Houghton et al., 1991; Houghton, 1996) and classified as a member of the flaviviridae family. Its genome is unsegmented and is approximately 9.5 kb in size with a large open-reading frame encoding a polyprotein of 3011 amino acids long. The genomes of structural proteins are located at the 5' end and the non-structural genes are at the 3' end. The structural proteins consist of a capsid protein and two envelope proteins (E1 and E2). The non-structural proteins consist of at least six polypeptides, including protease, helicase, RNA polymerase enzymes and regulatory peptides. HCV is not a homogeneous virus population but it comprises a heterogeneous group of viruses (Dusheiko et al., 1996). Current patterns of hepatitis C virus classification are based on genetic relatedness. As with many RNA viruses. HCV is highly diverged - at least six major genotypes and multiple subtypes have been described (Ohno and Lau, 1996).

HCV infection can be determined by detection of antibodies targeted to HCV structural and non-structural proteins. Serological tests such as enzyme linked immunoassays (ELISA) are used primarily to screen blood donors and for diagnosis of HCV infection in symptomatic patients.

The anti-HCV antibodies, even those detected by third generation methods, are presumably not the neutralising antibodies as they can be detected in the serum of patients with ongoing HCV infection (Koziel, 1996). Detection of HCV-RNA necessitates amplification of the circulating viral RNA, e.g. by using reverse transcriptase polymerase chain reaction (RT-PCR). Methods of quantitating HCV genomes in the serum are also available.

Hepatitis C vaccine is not yet available. Sequence variation especially in the envelope region of the HCV genome, high rates of mutation, and an obscure humoral response are all challenges for the development of an effective HCV vaccine (Katkov et al. 1996).

Hepatitis D virus (HDV) is a transmissible agent that is defective and requires co-infection with HBV for viral replication. Molecular studies led to the identification of the genome of HDV. It has a small genome (1700 nucleotides), consisting of single-stranded RNA, and was circular with a high degree of base pairing. The genome of HDV is present in a HD antigen nucleocapsid that is coated with HBsAg. Co-infection with this virus can be demonstrated by the presence of IgM antibodies to the delta agent (anti-HDV) in addition to

the markers of HBV infection. Co-infection with HBV and HDV may cause fulminant hepatitis. Super-infection with HDV is marked by the presence of both IgM and IgG antibodies directed to HDV together with negative or low titres of IgM anti-HBc. Super-infection may cause a deterioration of a previous stable chronic HBV carrier state and may lead to a more rapid progression of liver disease (Ramos-Soriano and Schwartz, 1994). HDV-RNA can be detected in the serum by the principle of RT-PCR or by other amplification methods (Ramos-Soriano and Schwartz, 1994).

Hepatitis E is an enterally transmitted disease, similar to hepatitis A, that is spread primarily by faecal contaminated drinking water (Dienstag and Isselbacher, 1994; Ramos-Soriano and Schwartz, 1994; Prudy and Krawzynski, 1994). HEV is small, spherical, non-enveloped virus with morphologic and biophysical properties most similar to viruses found in the family of Caliciviridae. The genome of HEV is approximately 7.5 kb in length and consists of a positive-sense, single stranded RNA molecule that contains three distinct open reading frames (ORF1, ORF2, ORF3) that appear to encode for non-structural and structural proteins based on the presence of well-defined consensus motifs and genomic organisation similar to those of other Calici- or Calici-like viruses (Reyes et al., 1990).

The precise taxonomy of the virus has not yet been clarified (Koziel, 1996). A number of diagnostic tests for HEV infection have been developed over recent years. Immune electron microscopy using serum taken after acute HEV infection was used to detect aggregates of viral particles in stool samples (Bradley et al., 1987). In specimens obtained by liver biopsy, the fluorescent antibody-blocking assay detects HEV antigen present in hepatocytes (Krawczynski and Bradley, 1989). The cloning of HEV (Reyes et al., 1990) has led to the development of a number of ELISAs using recombinant HEV antigens (Dawson et al., 1992; Tsarev et al., 1993). The assay was based on the detection of antibodies to the ORF2 region, which contains sequences thought to code for virus capsid protein, and the ORF3 region, which overlaps the ORF1 and ORF2 region of viral RNA (Reyes et al., 1990). Anti-HEV of the IgM class appears first in the serum and thereafter anti-HEV IgG. HEV-RNA can be detected by RT-PCR in the stool of patients in the acute phase of hepatitis E (Koziel, 1996).

Although sensitive and specific tests for detection of the known hepatitis viruses are available, there still have been nearly 20% of patients with acute hepatitis of which the

etiology remains unexplained (Deka et al., 1994; Alter HJ and Bradley, 1995). Indeed, a number of clinical studies have suggested the existence of additional viral agents responsible for the occurrence of hepatitis. These include sporadic hepatitis (Marcellin et al., 1993); a substantial fraction of post-transfusion hepatitis (Alter HJ et al., 1989); community-acquired acute resolving and chronic hepatitis (Alter MJ et al., 1992; Buti et al., 1994); and fulminant hepatitis (Fagan et al., 1992; Kuwada et al., 1994). These non-A, non-B, non-C, non-E cases are further referred to as non-A-E hepatitis.

In 1994, Deka and colleagues claimed to have transmitted an enteric agent (designated as HFV) responsible for sporadic non-A-E hepatitis from human to rhesus monkeys using a patient's stool extract (Deka et al., 1994). Virus-like particles, 27-37 nm in diameter, were reported to be present in the infectious human stool extract as well as in the liver and stools of inoculated monkeys. The particles were shown to contain about 20 kilobases of double-stranded DNA. This finding has unfortunately not yet been confirmed by others until to date. More extensive studies will be required to confirm the existence of HFV.

In 1967, Deinhardt et al. Published serial transmission experiments in animal models with suspected infectious sera of patients with hepatitis of unknown etiology (Deinhardt et al., 1967). One of the infectious sera was obtained from a 34 year old surgeon with initials GB, collected during the third day of jaundice. He had no obvious exposure to serum hepatitis and had a history of moderate illness. Five serial marmoset-to-marmoset passages were carried out, and resulted in serum elevations of liver enzymes and histopathological changes in liver biopsies of almost all inoculated animals. The identification of the hepatitis A and B viruses in 1970's has excluded these two viral agents as the cause of hepatitis in this surgeon. Subsequent experiments have led to the further characterisation of the "hepatitis GB agent" (Almeida et al., 1976; Karayiannis et al., 1989).

Simons et al. have successfully cloned two flavivirus-like genomes from sera of tamarinds infected with the GB hepatitis agent (Simons et al., 1995a). Phylogenetic analysis showed that the sequences, which were designated as GB virus A and GB virus B, are neither genotypes of HCV nor genotypes of the same virus.

The genomes of GBV-A and GBV-B consist of 9493 nucleotides and 9143 nucleotides respectively (Muerhoff et al., 1995). Northern blot hybridisation showed that RNAs of GBV-B

but not of GBV-A could be detected in the liver of the inoculated tamarinds. GBV-B viremia caused a serum elevation of liver enzymes, whereas no enzyme elevation was observed in animals with only GBV-A viremia. Although nucleic acids of both viruses were present in plasma in acute phase, the exclusive evidence for the present of GBV-B in livers of infected animals suggests that GBV-B causes hepatitis in the inoculated tamarinds, while GBV-A seems only to be a transmissible virus in tamarinds without hepatitis inducing capacity by itself. However, further in vivo studies by Schlauder et al. (1995) demonstrated that the peaks of ALT-values of GBV-B infected animals were significantly lower than in GBV-A/GBV-B co-infected animals, suggesting that the severity of hepatitis in these animals is related to the presence of both viruses.

Efforts were subsequently made to detect antibodies in human sera by ELISA using recombinant proteins generated from GBV-A and GBV-B. Numerous immunoreactive sera were identified, but studies with RT-PCR using specific primers for these agents failed to confirm the presence of viral nucleic acids. The positive results of serological investigations might therefore have been due to cross-reactivities with proteins encoded by a related virus. Further investigations pointed out that GBV-A induces hepatitis in tamarinds but has probably no relevance in human (Schlauder et al., 1995).

Despite the above mentioned findings, further investigation of those immunoreactive sera with degenerate primers of the putative NS3 helicase region revealed a RT-PCR product with limited sequence identity to GBV-A, GBV-B and HCV. GENBANK analysis and alignment of the predicted translation product to other positive stranded RNA-viruses indicated that this sequence is derived from a novel member of the flaviviruses. This new virus was designated as GB virus C (GBV-C). The GBV-C viral genome consists of a positive sense, single-stranded RNA molecule of 9.2 kb. GBV-C encodes a putative single large polyprotein in which the structural proteins are positioned at the N-terminal end, and the non-structural proteins at the C-terminal end. Sequence analysis revealed that GBV-C was 59.0%, 47.9% and 53.7% identical at the nucleotide level, and 64.2%, 50.4% and 57.3% identical at amino acid level to GBV-A, GBV-B and HCV-1 sequences, respectively. These findings indicated that GBV-C is a member of the Flaviviridae family, more closely related to GBV-A than to GBV-B and clearly distinct from the representative member of the HCV family.

Parallel to the discovery of the hepatitis GB viruses, another flavivirus-like agent was identified. In January 1996, Linnen et al. reported the identification of an RNA virus that was designated as hepatitis G virus (Linnen et al., 1996). Further studies using an immunoreactive complementary DNA clone yielded the entire genome of 9392 nucleotides encoding a polyprotein of 2873 amino acids. The virus is closely related to GBV-C and distantly related to HCV, GBV-A, and GBV-B. This virus is a member of the flavivirus family and is associated with acute and chronic hepatitis in humans. This virus was detected in five of 38 patients (13%) with acute non-A-E hepatitis. None of the five patients developed chronic hepatitis but four remained HGV-RNA positive over a period of 2 to 9 years. In addition, HGV-RNA was detected in the serum of 18% of patients with acute hepatitis C, 1.7% of blood donors with normal serum alanine aminotransferase (ALT) levels, and 1.5% of blood donors with elevated ALT levels (>45 IU/L) (Linnen et al., 1996). The virus is found also to associate with both sporadic and post-transfusion hepatitis (Simons et al., 1995b; Linnen et al., 1996). HGV has also been detected in the serum of patients with non-A-E fulminant hepatitis and in patients with chronic non-A-E hepatitis (Yoshiba et al., 1995; Dawson et al., 1996). It has a global distribution. Nevertheless, the role of this virus in the pathogenesis of liver injury is still not clear (Alter MJ et al., 1996).

From these findings, it is clear that HGV-RNA was not detected in many acute and chronic non-A, B, C cases even when using three different primer sets which are derived from the 5'-UTR, NS3 and NS5 regions of the HGV genome.. While the current assays may lack sensitivity, it is more likely that such cases are due to other hepatotropic agents. GBV-B may be responsible for some of the acute non-A-E hepatitis cases, but it seems that this viral agent is not associated with chronic hepatitis (Alter HJ et al., 1997; Alter MJ et al., 1997; Yashina et al., 1997).

Recently, the putative envelope protein (E2) of HGV has been cloned and expressed in Chinese hamster ovary cells (Tacke et al., 1997; Dille et al., 1997). This E2 viral protein is probably located on the surface of the virus, and has been assumed to be a target for human immune responses. An ELISA for detection of anti-HGV-E2 antibodies has been developed. Detection of antibodies to this protein is thought to be associated with a past HGV infection (Tacke et al., 1997; Dille et al. 1997).

In December 1997, a novel DNA virus was reported by Nishizawa et al. to be associated with elevated aminotransferase levels in the sera of 3 to 5 patients with biopsy-proven post-transfusion hepatitis of an at that time unknown aetiology. Association with elevated aminotransferase levels was reported by Nishizawa et al. This virus was designated TT-virus (TTV), after the initials of the first patient from whom the virus was isolated. TTV is an unenveloped single-stranded DNA virus for which a sequence of 3,739 bases was determined.

In subsequent analyses, evidence of potential hepatotropism of TTV was reported with TTV-DNA titers shown to be 10- to 100-fold greater in liver tissue than in serum. In addition, TTV-DNA titers correlated with aminotransferase levels in TTV-infected patients with non-A-G post-transfusion hepatitis. The relative hepatotropism and correlation of TTV titers with biochemical markers of hepatitis are in contrast to HGV, for which neither characteristic has been demonstrated.

In follow-up to the initial description of TTV (Okamoto et al., 1998 it was reported that 12% (34 of 290) of Japanese blood donors have detectable TTV DNA in their serum as measured by PCR. The prevalence of TTV in Japanese patients with chronic non-A-G hepatitis (and/or cirrhosis) was found to be 46% (41 of 90). Similar prevalences were reported for patients with risk factors for parenteral transmission of infectious agents, including intravenous drug users (40%), patients with haemophilia A (68%), and hemodialysis patients (46%). Clearly, with prevalences of this magnitude, TTV infection can produce liver disease in a minority of, if any, infected patients. However, it is possible that in a subset of TTV-infected patients, TTV may cause acute and/or chronic liver injury. This may be on the basis of host or viral characteristics, such as genotypic variations in TTV.

Two characteristics of post-transfusion TTV-infection make TTV a relatively attractive candidate virus as a potential cause of liver disease. The first of these is the observation that in TTV-infected patients with non-A-E post-transfusion hepatitis, the concentration of TTV is on average 10- to 100-fold higher in liver tissue than in serum. The second characteristic is that, in newly TTV-infected patients with non-A-E post-transfusion hepatitis, TTV titres rose and fell with aminotransferase levels, becoming undetectable in patients who normalised their aminotransferases. Neither of these characteristics, hepatotropism or correlation of viral titers with serum transaminases, were ever demonstrated for HGV. It should be kept in mind, however, that the higher prevalence of TTV infection in patients with cryptogenic cirrhosis

and idiopathic fulminant hepatic failure may simply reflect higher rates of parenteral and non-parenteral exposure to TTV than was experienced by the control groups.

As these recently described viruses at best seem to account for only a part of the non A-E hepatitis cases, it will be advantageous if means would be available to detect and characterise other non A-E hepatitis causing agents. This will allow for the development of specific diagnostic tests, anti-viral agents and preventive and therapeutic vaccines.

We undertook a prospective study in 165 patients who were admitted to the liver unit of the University Hospital in Leuven, Belgium for a liver biopsy because of acute or chronic liver disease. These patients underwent hepatological work-up including determination of serological markers of known hepatitis virus infection, autoantibodies for the diagnosis of autoimmune liver disease and tests for metabolic liver disease such as haemochromatosis and Wilson's disease. Data on anti-HCV and serum HCV-RNA were also collected from the past examinations and during follow-up in order to exclude intermittent viremia which may exist in seronegative chronic hepatitis C patients. In addition, a possible infection with HGV was also investigated by testing serum HGV-RNA using a PCR-assay. Liver biopsies were reviewed for histology and examined for granular immunoreactivity to the monoclonal antibody HCV.OT1F as described previously (Yap et al.,1994 , Example 1).

In summary, in this prospective study of 165 patients we have identified a large number of non-A-E, non-G hepatitis patients further referred to as non-A-G hepatitis patients (Example 1). Interestingly, liver biopsies from a large number of 121 patients from this study showed immunoreactivity with monoclonal antibody HCV-OT1F. When all HCV patients were excluded from this group, a number of 93 patients remained. This finding was unexpected since monoclonal antibody HCV-OT1F was raised against an amino acid sequence derived from HCV core antigen and previously thought to be rather specific for HCV (Yap et al.,1994). When this finding was further investigated it appeared that monoclonal antibody HCV-OT1F was capable of reacting with many more amino acid sequences than just the amino acid sequence of HCV against which it was raised (Example 3). Data obtained from a study based on a so-called replacement net of the HCV sequence against which the monoclonal antibody HCV-OT1F was raised, confirm this notion. Since the group

of non-A-G patients largely overlaps with the group of patients positive with monoclonal antibody HCV-OT1F but negative for any signs of HCV infection, it is concluded that immunological staining with monoclonal antibody HCV-OT1F might provide a useful tool in the diagnosis of a new form of non-A-G viral hepatitis.

The immunostaining method with monoclonal antibody HCV.OT1F also provides for a tool to detect transmission of such a new form of non-A-G viral hepatitis as it may occur in patients with a persisting hepatitis after orthotopic liver transplantation (OLT) who were liver HCV.OT1F immunoreactive, but remained serum HCV-RNA and anti-HCV negative during follow-up. A review of the viral and histological data of these patients before OLT, showed that HCV-OT1F liver immunoreactivity was found prior to OLT, despite anti-HCV and serum HCV-RNA were negative and remained negative after OLT. Finally, in one patient, HCV-OT1F immunoreactivity associated with chronic hepatitis was found after OLT while it was negative before OLT. This finding is strongly suggestive for a *de novo* infection, possibly by the route of blood transfusion. From these findings based on clinical studies of patients who underwent OLT, we can therefore conclude that anti-HCV negative, serum HCV RNA negative and liver HCV-OT1F immunoreactivity associated liver disease is caused by a transmissible agent (Example 2).

Disease could also be transmitted by inoculation of non-human primates like chimpanzee and rhesus monkey with serum, plasma, liver cells and/or PBMC obtained from a human patient or of other infected non-human primates (Example 4).

In the material obtained from the above non-A-G patients we discovered and characterised a new etiological hepatitis agent and termed it Hepatitis Y virus (HYV).

The invention described here provides nucleic acids and polypeptides derivable from the hepatitis Y etiological agent HYV. Moreover, the present invention also provides tools for diagnosing an infection with this virus in patients suspected of having a viral hepatitis as well as vaccines for the prevention of disease. The invention also provides a method for the in vitro culturing of HYV, as well as methods for determining the presence of the nucleic acid of this agent in materials like plasma, serum and liver tissue or isolated liver cells from HYV infected individuals or non-human primates like chimpanzee or rhesus monkey as well as from culture

supernatants or cell lysate of in vitro infected cell cultures. The invention also provides antigens derivable from the genome of hepatitis Y and antibodies reacting to these antigens.

The invention provides nucleic acids derivable from the genome of HYV. These sequences were obtained as described in Example 6. In brief: a cDNA difference analysis protocol, essentially as described by Hubank M. and Schatz, D. (NAR,22,5640-5648) is followed using nucleic acid from serum obtained from (a) non-A-G hepatitis patient(s) using human cot-1-DNA (Gibco), liver cDNA and human genomic DNA as the so-called driver in successive rounds of differential hybridisation (Example 6). Alternatively, plasma or liver from non-A-G infected patients may be used as a source of nucleic acid for this procedure. Enriched sequences were cloned into a vector and grown in an appropriate bacterial system to allow sequencing of the insert. Excluding known sequences, primers for PCR were designed for the sequences obtained. PCR applied to cDNA prepared from material including serum, plasma and liver cells from patients suspected to be infected with Hepatitis Y and controls allowed specific detection of hepatitis Y and isolation of further sequences specific for HYV. As an example of such sequences SEQ ID NO: 1 and SEQ ID NO: 2 are provided. (Example 6). Plasmids containing this sequence were deposited at the ECACC (accession numbers 98121504 and 98121505).

SEQ ID NO: 1:

```
GATCACAAGC AACTGCCCGA CGAACGTACG CTGAGCGTAT TCGTCGACGA ACTGCACGCC      60

CTCGACAAAC AGCGCCTGTC CGGCAAGCTG TCCGAGGAGT TCAACCGCGC CTATACCGGC     120

ATGTCCAGCG TGGCCAAAGC CACTGCCCGG CGCGTTGGCC GACTGGACGC CCAGGCGCTG     180

CAAAGCCAAG GCGTGCAGAC GCTGCTCGAG GCCCACCGCA ACTGGAGCAA GCCCGAGCTG     240

TGGTACGCCA TCGAGCGCGC CGGCAAGGTT TACACCTACG ATTACTACCT GACCGCACTG     300

GATC                                                                  304
```

SEQ ID NO: 2

GATCGABGTG CAACACGCCC GCCTATHACG GCGCGTATTG CTTGTBGCAG CCTGAGTGCA 60

GCATTAGATT AGCCAATTAT CTGGGGCACC ATCATAAGCA GAAGGGATAA GCATGGCGCT 120

CACCGACCAA TCCACCCGCA CCCGTACCGG CGAAGAACTC GACGCTGCCG TCATCGACGC 180

CTATCTCAAG GCCcATATTC CCGGCCTGAG TGGCGAGGCC GG 222

NOTE: in SEQ ID NO: 2, B = C, G or T; H = A, C or T; c = probably C

Cesium chloride equilibrium gradient centrifugation fractions were prepared from serum of a patient with Hepatitis Y or from high speed centrifugation-supernatant of liver homogenate from another patient with Hepatitis Y. Both were tested by nucleic acid amplification using nested primers according to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO:6 derived from SEQ ID NO: 1. Fractions with a density around 1.22 g/cm3 were found to contain genomic sequences detectable by the method described in Example 7. These fractions thus contain the purified virus particles. Accordingly, the invention provides a Hepatitis Y virus characterized in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement.

Nthe invention thus provides a nucleic acid sequence derivable from the genome of Hepatitis Y virus or from fragments of said genome or functional equivalents of said nucleic acid sequence, wherein Hepatitis Y virus is characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement.

Having available a probe for HYV as provided with SEQ ID NO: 1 or SEQ ID NO: 2, methods to characterize the HYV particle and its genomic nucleic acid are now further routine in the field and include sucrose gradient centrifugation to determine sedimentation coefficient, ultra filtration through controlled pore filters to determine particle size, disruption with detergents and chaotropic agents to determine composition of the particle.

The HYV Genome

Further polynucleotides from the genome of HYV may be obtained by routine methods, for example by producing a phage library from nucleic acid isolated from serum, plasma or liver from HYV positive humans or non-human primates or cell culture extracts and plate these phages to form plaques. The resulting phages can than be screened using SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement or fragments thereof as hybridization probes. Other hybridization probes or PCR primers can then easily be selected.

Hybridization probes and primers for nucleic acid amplification methods are also part of the invention. The use of these for the further characterization of the genome of Hepatitis Y etiological agent is described in Example 9. For this purpose a total nucleic acid preparation created by hexamer primed first- and second strand cDNA synthesis on serum nucleic acids, tissue culture extracts or purified viral material using standard procedures is used as input material.

In a first round this preparation is subjected to a-specific amplification by anchor primer addition through blunt-end ligation in which the anchor primer has a specific restriction site and a subsequent PCR reaction with the anchor primer. After restriction digestion of the PCR products, these products are cloned in a phage vector and plaques created with the recombinant phages are screened by hybridization with a HYV specific probe.

Alternatively, anchor linkers, consisting of a long and a short arm that are both unphosphorylated, can be added to the ends of the cDNA products after restriction digestion with a restriction enzyme compatible with the overhang of these primers. After non-specific amplification with anchor-complementary primers specific amplification can be performed by ligating these PCR products in a cloning vector (by specific restriction or through T/A cloning) and performing a PCR reaction on this ligation reaction using a combination of vectorprimers situated on both sides of the insert and one of two primers situated on the complementary strand of a double stranded HYV specific sequence. The resulting PCR products can be enriched further for HYV specific sequences by ligating these PCR products in a vector and performing a PCR on this ligation reaction using a combination of vector primers situated on both sides of the insert and one of two primers situated on the

complementary strand of a double stranded HYV specific sequence but downstream of the primers in the preceding PCR reaction. These PCR products can be cloned, screened with HYV specific primers situated within the region bounded by the restriction site used and the HYV specific primer used in the final amplification step.

Another approach is by making use of the Marathon cDNA procedure (Clontech). In this procedure double stranded cDNA is ligated with a specially designed adaptor sequence of which the shorter sequence is blocked. This sequence will therefore not be elongated and is displaced by the product made by elongating the HYV specific primer. The resulting PCR products can be screened further by one of the procedures above or a combination of these.

Segmentation of the genome can be detected by Northern blotting. If the viral genome is unsegmented, the complete genome can be isolated applying one or more of the procedures described above.

The extreme 5'- and 3'-ends of the genome are isolated by ligating the genomes head to tail together and isolating the junction fragments by PCR using as primers the sequences situated on the ends of the established sequence.

If the viral genome is segmented, and on the assumption that different segments have sequence motif in common, cDNA synthesis and PCR amplification as described above is followed by cloning in a vector and hybridization screening with the complete HYV specific sequence.

Otherwise, if the segments do not have motifs in common, a new RDA experiment can be performed using the known HYV sequence as complementary driver sequence.

HYV encoded Peptides

The invention also provides polypeptides encoded by the HYV genome. From the sequences of SEQ ID NO: 1 the potential amino acid sequence can be read in six frames as provided in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 and SEQ ID NO: 16

SEQ ID NO 11:

```
Ser Gln Ala Thr Ala Arg Arg Thr Tyr Ala Glu Arg Ile Arg Arg Arg
 1               5                  10                  15

Thr Ala Arg Pro Arg Gln Thr Ala Pro Val Arg Gln Ala Val Arg Gly
            20                  25                  30

Val Gln Pro Arg Leu Tyr Arg His Val Gln Arg Gly Gln Ser His Cys
        35                  40                  45

Pro Ala Arg Trp Pro Thr Gly Arg Pro Gly Ala Ala Lys Pro Arg Arg
    50                  55                  60

Ala Asp Ala Ala Arg Gly Pro Pro Gln Leu Glu Gln Ala Arg Ala Val
65                  70                  75                  80

Val Arg His Arg Ala Arg Arg Gln Gly Leu His Leu Arg Leu Leu Pro
                85                  90                  95

Asp Arg Thr Gly
            100
```

SEQ ID NO: 12:

```
Ile Thr Ser Asn Cys Pro Thr Asn Val Arg Xaa Ala Tyr Ser Ser Thr
 1               5                  10                  15

Asn Cys Thr Pro Ser Thr Asn Ser Ala Cys Pro Ala Ser Cys Pro Arg
            20                  25                  30

Ser Ser Thr Ala Pro Ile Pro Ala Cys Pro Ala Trp Pro Lys Pro Leu
        35                  40                  45

Pro Gly Ala Leu Ala Asp Trp Thr Pro Arg Arg Cys Lys Ala Lys Ala
    50                  55                  60

Cys Arg Arg Cys Ser Arg Pro Thr Ala Thr Gly Ala Ser Pro Ser Cys
65                  70                  75                  80

Gly Thr Pro Ser Ser Ala Pro Ala Arg Phe Thr Pro Thr Ile Thr Thr
                85                  90                  95

Xaa Pro His Trp Ile
            100
```

SEQ ID NO: 13:

Asp His Lys Gln Leu Pro Asp Glu Arg Thr Leu Ser Val Phe Val Asp
1 5 10 15

Glu Leu His Ala Leu Asp Lys Gln Arg Leu Ser Gly Lys Leu Ser Glu
20 25 30

Glu Phe Asn Arg Ala Tyr Thr Gly Met Ser Ser Val Ala Lys Ala Thr
35 40 45

Ala Arg Arg Val Gly Arg Leu Asp Ala Gln Ala Leu Gln Ser Gln Gly
50 55 60

Val Gln Thr Leu Leu Glu Ala His Arg Asn Trp Ser Lys Pro Glu Leu
65 70 75 80

Trp Tyr Ala Ile Glu Arg Ala Gly Lys Val Tyr Thr Tyr Asp Tyr Tyr
85 90 95

Leu Thr Ala Leu Asp
100

SEQ ID NO: 14:

Asp Pro Val Arg Ser Gly Ser Asn Arg Arg Cys Lys Pro Cys Arg Arg
1 5 10 15

Ala Arg Trp Arg Thr Thr Ala Arg Ala Cys Ser Ser Cys Gly Gly Pro
20 25 30

Arg Ala Ala Ser Ala Arg Leu Gly Phe Ala Ala Pro Gly Arg Pro Val
35 40 45

Gly Gln Arg Ala Gly Gln Trp Leu Trp Pro Arg Trp Thr Cys Arg Tyr
50 55 60

Arg Arg Gly Xaa Thr Pro Arg Thr Ala Cys Arg Thr Gly Ala Val Cys
65 70 75 80

Arg Gly Arg Ala Val Arg Arg Arg Ile Arg Ser Ala Tyr Val Arg Arg
85 90 95

Ala Val Ala Cys Asp
100

SEQ ID NO: 15:

Ile Gln Cys Gly Gln Val Val Ile Val Gly Val Asn Leu Ala Gly Ala
1 5 10 15

Leu Asp Gly Val Pro Gln Leu Gly Leu Ala Pro Val Ala Val Gly Leu
20 25 30

Glu Gln Arg Leu His Ala Leu Ala Leu Gln Arg Leu Gly Val Gln Ser
35 40 45

Ala Asn Ala Pro Gly Ser Gly Phe Gly His Ala Gly His Ala Gly Ile
50 55 60

Gly Ala Val Glu Leu Leu Gly Gln Leu Ala Gly Gln Ala Leu Phe Val
65 70 75 80

Glu Gly Val Gln Phe Val Asp Glu Tyr Ala Gln Arg Thr Phe Val Gly
85 90 95

Gln Leu Leu Val Ile
100

SEQ ID NO: 16:

Ser Ser Ala Val Arg Xaa Xaa Ser Xaa Val Xaa Thr Leu Pro Ala Arg
1 5 10 15

Ser Met Ala Tyr His Ser Ser Gly Leu Leu Gln Leu Arg Trp Ala Ser
20 25 30

Ser Ser Val Cys Thr Pro Trp Leu Cys Ser Ala Trp Ala Ser Ser Arg
35 40 45

Pro Thr Arg Arg Ala Val Ala Leu Ala Thr Leu Asp Met Pro Val Xaa
50 55 60

Ala Arg Leu Asn Ser Ser Asp Ser Leu Pro Asp Arg Arg Cys Leu Ser
65 70 75 80

Arg Ala Cys Ser Ser Ser Thr Asn Thr Leu Ser Val Arg Ser Ser Gly
85 90 95

Ser Cys Leu Xaa
100

NOTE: in SEQ ID NO 11 -SEQ ID NO:16 Xaa denotes a termination codon

Other peptides may easily be obtained by expression cloning. In this method, phages are prepared from a library as described above using the methods of Huynh et al. (In: D. Glover (ed.), DNA Cloning: A Practical Approach, IRL Press Oxford, 49-78, 1985). The phages are plated to form plaques and the plaques are screened for production of poly-peptides immunoreactive with HYV serum. The serum used for screening the plaques can be from any HYV source including human, chimpanzee or rhesus monkey serum. Alternatively, immunogenic oligopeptides may be deduced from the nucleic acid sequences obtained as described above by methods widely known in the field. Once an amino acid sequence is known, immunogenic and antigenic fragments thereof may be obtained by methods described below. From the nucleic acid sequences SEQ ID NO:1 or the sequence of SEQ ID NO: 2 or other HYV sequences, one or more reading frames can be deduced. Overlapping oligopeptides can be synthesized and screened for immunoreactivity with HYV sera.

Thus, the invention provides a polypeptide comprising an amino acid sequence or fragment thereof wherein said amino acid sequence is encoded by a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement, or any functional equivalents of said polypeptide.

Also included within the scope of the present invention are nucleic acid sequences derivable from functional variants of (fragments of) the HYV genome as well as functional variants of the encoded amino acid sequences as for instance are shown in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 or SEQ ID NO: 16.

It will be understood that for a particular polypeptide derived from HYV, natural variations can exist between individual viruses or strains. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions from which can be expected that they do not essentially alter biological and immunological activities, have been described. Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and

structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Based on this information Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science 227, 1435-1441, 1985) and determining the functional similarity between homologous polypeptides. Nucleic acid sequences encoding such homologous functional variants are included within the scope of this invention. Moreover, the potential exists to use recombinant DNA technology for the preparation of nucleic acid sequences encoding these various functional variants.

The information provided in SEQ ID NO: 1 or SEQ ID NO:2 allows a person skilled in the art to isolate and identify the nucleic acid sequences encoding the various functional variant polypeptides mentioned-above having corresponding immunological characteristics with the protein specifically disclosed herein. The generally applied Southern blotting technique or colony hybridization can be used for that purpose (Experiments in Molecular Biology, ed. R.J. Slater, Clifton, U.S.A., 1986; Singer-Sam, J. et al., Proc. Natl. Acad. Sci. 80, 802-806, 1983; Maniatis T. et al., Molecular Cloning, A laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, USA, 1989). For example, restriction enzyme digested DNA fragments derived from a specific HYV strain is electrophoresed and transferred, or "blotted" thereafter onto a piece of nitrocellulose filter. It is now possible to identify the nucleic sequences encoding the functional variant polypeptides on the filter by hybridization to a defined labelled DNA or "probe" back translated from the amino acid sequences shown in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 or SEQ ID NO: 16, under specific conditions of salt concentration and temperature that allow hybridization of the probe to any homologous DNA sequences present on the filter. After washing the filter, hybridized material may be detected by autoradiography. Once having identified the relevant sequence, DNA fragments that encode a functionally variant polypeptide to the polypeptide disclosed in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 or SEQ ID NO: 16 can now be recovered after agarose gel electrophoresis.

A nucleic acid sequence according to the invention may be isolated from a particular HYV strain and multiplied by molecular biology methods including recombinant DNA techniques and polymerase chain reaction (PCR) technology or may be chemically synthesized in vitro by techniques known in the art.

A preferred nucleic acid sequence according to the invention is characterized in that said sequence contains at least part of the DNA sequence disclosed in SEQ ID NO:1 or its complement.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in an other codon but still coding for the same amino acid, e.g. both GAT and GAA are codons for the amino acid glutamic acid. Consequently, it is clear that for the expression of a polypeptide with the amino acid sequence shown in SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO:14, SEQ ID NO: 15 or SEQ ID NO: 16 use can be made of a variant nucleic acid sequence with such an alternative codon composition different from the nucleic acid sequence shown in SEQ ID NO:1.

Furthermore, also fragments of the nucleic acid sequences encoding the specifically disclosed protein or functional variants thereof as mentioned above are included in the present invention. All modifications resulting in the above-mentioned functional variants of the specifically exemplified polypeptide are included within the scope of the present invention.

The term "fragment" as used herein means a DNA or amino acid sequence comprising a subsequence of the nucleic acid sequence or polypeptide of the invention. Said fragment is or encodes a polypeptide having one or more immunogenic determinants of a particular HYV protein, i.e. has one or more epitopes of the HYV protein reactive with a given antibody or capable of eliciting an immune response in a suitable host. Fragments can inter alia be produced by enzymatic cleavage of precursor molecules, using restriction endonucleases for the DNA and proteases for the polypeptides. Other methods include chemical synthesis of the fragments or the expression of polypeptide fragments by DNA fragments.

An "immunogenic fragment" is understood to be a fragment of the full-length protein that has retained its capability to induce an immune response in the host. An "immunoreactive fragment" is understood to be a fragment of the full length protein that has retained its capability to react with an antibody. At this moment, a variety of techniques is available to easily identify DNA fragments encoding immunogenic or immunoreactive fragments (determinants). The method described by Geysen et al (Patent Application WO 84/03564, Patent Application WO 86/06487, US Patent nr. 4,833,092, Proc. Natl Acad. Sci. 81: 3998-

4002 (1984), J. Imm. Meth. 102, 259-274 (1987), the so-called PEPSCAN method is an easy to perform, quick and well-established method for the detection of epitopes; the immunologically important regions of the protein, used world-wide and as such well-known to man skilled in the art. This (empirical) method is especially suitable for the detection of B-cell epitopes. Also, given the sequence of the gene encoding any protein, computer algorithms are able to designate specific polypeptide fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are now known. The determination of these regions is based on a combination of the hydrophilicity criteria according to Hopp and Woods (Proc. Natl. Acad. Sci. 78: 38248-3828 (1981)), and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 47: 45-148 (1987) and US Patent 4,554,101). T-cell epitopes can likewise be predicted from the sequence by computer with the aid of Berzofsky's amphiphilicity criterion (Science 235, 1059-1062 (1987) and US Patent application NTIS US 07/005,885). A condensed overview is found in: Shan Lu on common principles: Tibtech 9: 238-242 (1991), Good et al on Malaria epitopes; Science 235: 1059-1062 (1987), Lu for a review; Vaccine 10: 3-7 (1992), Berzowsky for HIV-epitopes; The FASEB Journal 5:2412-2418 (1991)

Two nucleic acid fragments are considered to have hybridisable sequences if they are capable to hybridising to one another under typical hybridisation and wash conditions, as described, for example in Maniatis, et al., pages 320-328, and 382-389, or using reduced stringency wash conditions that allow at most about 25-30% basepair mismatches, for example: 2x SSC, 0.1% SDS, room temperature twice, 30 minutes each, then 2x SSC, 0.1% SDS 37 °C once, 30 minutes; then 2X SSC, room temperature twice ten minutes each. Preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches. These degrees of homology can be selected by using wash conditions of appropriate stringency for identification of clones from gene libraries or other sources of genetic material, as is well known in the art.

Two amino acid sequences or two nucleic acid sequences are considered homologous if they have an alignment score of more than 5 (in standard deviation units) using the program ALIGN with the mutation gap matrix and a gap penalty of 6 or greater (Dayhoff). The two sequences (or parts thereof, preferably at least 35 amino acids in length) are homologous if

their amino acid alignments are greater than or equal to 40% preferably 60% and more preferably 80% using the ALIGN program mentioned above.

A DNA or cDNA fragment is derivable from the Hepatitis Y genome if it has the same or substantiall the same basepair sequence as a cloned region of a Hepatitis Y genome

Antibodies against HYV

Polyclonal and monoclonal antibodies, either purified from a natural host including human, chimpanzee or rhesus monkey infected by HYV or raised in experimental animals against HYV polypeptides or other structural elements are also part of the invention.

Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogeneous binding characteristics for the relevant antigen. Homogeneous binding as used herein refers to the ability of the antibody species to bind to ligand binding domain of the invention.

The antibody is preferably a monoclonal antibody, more preferably a humanised monoclonal antibody.

Monoclonal antibodies can be prepared by immunizing inbred mice, preferably Balb/c with the appropriate protein by techniques known in the art (Kohler and Milstein, Nature 256; 495-497, 1975). Hybridoma cells are subsequently selected by growth in hypoxanthine, thymidine and aminopterin in an appropriate cell culture medium such as Dulbecco's modified Eagle's medium (DMEM). Antibody producing hybridomas are cloned, preferably using the soft agar technique of MacPherson, (Soft Agar Techniques, Tissue Culture Methods and Applications, Kruse and Paterson, eds., Academic Press, 276, 1973). Discrete colonies are transferred into individual wells of culture plates for cultivation in an appropriate culture medium. Antibody producing cells are identified by screening with the appropriate immunogen. Immunogen positive hybridoma cells are maintained by techniques known in the art. Specific monoclonal antibodies are produced by cultivating the hybridomas in vitro or preparing ascites fluid in mice following hybridoma injection by procedures known in the art.

It is preferred to use humanized antibodies. Methods for humanizing antibodies, such as CDR-grafting, are known (Jones et al., Nature 321, 522-525, 1986). Another possibility to

avoid antigenic response to antibodies reactive with polypeptides according to the invention is the use of human antibodies or fragments or derivatives thereof.

Human antibodies can be produced by in vitro stimulation of isolated B-lymphocytes, or they can be isolated from (immortalized) B-lymphocytes which have been harvested from a human being infected with HYV or immunized with at least one polypeptide according to the invention.

It is also preferred to use single chain antibodies. Methods for the production of such antibodies are known in the art.

The invention thus provides antibodies reactive with a polypeptide encoded by the genome of hepatitis Y virus or functional equivalents thereof

Diagnostic assays for HYV

Diagnostic assays based on the use of nucleic acid amplification and/or nucleic acid hybridization are also part of the invention. The primers and probes can be designed using methods known in the art, for instance based on SEQ ID NO: 1, SEQ ID NO: 2 or other sequences derived from DNA sources as described in Example 7 and 9. An example of a set of primers based on SEQ ID NO:1 is given in SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO:6, examples of primers based on SEQ ID NO: 2 are provided in SEQ ID NO: 7, SEQ ID NO: 8 ,SEQ ID NO:9. and SEQ ID NO: 10,

SEQ ID NO: 3	CGTACGCTGAGCGTA
SEQ ID NO: 4	GGCGTACCACAGCTC
SEQ ID NO: 5	CACGCCCTCGACAAACAGCG
SEQ ID NO: 6	TCTGCACGCCTTGGTTTGCA
SEQ ID NO: 7	GTGCAACACGCCCGC
SEQ ID NO: 8	CCGGCCTCGCCACTC
SEQ ID NO: 9	ACGGCGCGTATTGCTTGT
SEQ ID NO: 10	GCCGGGAATATGGGCCTT

The method described in Example 7 typically allows detection of the HYV genome in clinical samples like serum or plasma. The results show that 7 out of 10 non-A-G patients were positively identified as carrying the HYV genome in their serum or liver. Zero out of 6 control subjects were positive. More detais are given in Example 7.

Standard methods of epitope prediction can be used to select the most likely immunoreactive regions as described herein. Synthetic peptides representing immunogenic, immunoreactive or functional domains of the HYV related polypeptides can be prepared by standard methods known in the art. Such oligopeptides as well as polypeptides produced by expression of recombinant DNA in prokaryotic or eukaryotic systems are also part of the invention

Another preferred embodiment of the present invention is directed to a method for the detection of antibodies to HYV, the method comprising contacting the sample suspected to contain antibodies to hepatitis Y virus particles or antigens with at least one peptide according to the present invention.

Yet another preferred embodiment of the present invention is directed to the detection of nucleic acid from HYV or related to HYV. This method may comprise contacting the sample with a suitable nucleic acid probe or probes and detecting binding of the probe or probes. Alternatively, the nucleic acid from the sample may first be amplified in a specific or non-specific way. Many suitable methods for these purposes are known in the art.

In *in vitro* assays, some form of supports is often used to immobilise the binding molecules or peptides according to the present invention. Supports which can be used are, for example, the inner wall of a microtest well or a cuvette, a tube or capillary, a membrane, filter, test strip or the surface of a particle such as, for example, a latex particle, an ceramic particle (such as a ceramic magnetizable particle with active aldehyde surface groups), an erythrocyte, a dye sol, a metal sol or metal compound as sol particle, a carrier protein such as BSA or KLH.

Also some form of labeling is often used to detect the antigen-antibody or hybridisation interaction. Labeling substances may be radioactive or non-radioactive such as a radioactive isotope, a fluorescent compound, a chemiluminescent compound, an enzyme, a dye sol, metal sol or metal compound as sol particle. Depending upon the format of the assay, either the specific binding molecules within the scope of the invention can be labeled, or other specific binding molecules, which bind to them are labeled. Immunoassays (including radioimmunoassays) and immunometric assays (including immunometric radioassays and enzyme-linked immunosorbent assays) can be used, as can immunoblotting techniques.

In vitro assays may take many formats. Some depend upon the use of labeled specific binding molecules such as antibodies (whose use is included within the scope of the invention), whereas some detect the interaction of antibody (or other specific binding molecule) and antigen by observing the resulting precipitation. These are well known in the art.

In vitro assays will often be conducted using kits. According to yet another aspect of the present invention, there is provided an assay kit for the detection of hepatitis Y viral particle or antigen, the kit comprising a specific binding molecule as described above and means for detecting whether the specific binding molecule is bound to a hepatitis Y particle, antigen or nucleic acid.

The assay methodology may for example be any of the assays referred to above. Competitive and, especially, sandwich immunoassay kits are preferred. The specific binding molecule and the detection means may be provided in separate compartments of the kit. The specific binding molecule may be provided bound to a solid support. The detection means may comprise a detectable labeled second specific binding molecule (which itself may be an antibody (monoclonal but preferably polyclonal), which bind to the bound hepatitis Y particle or antigen.

Also preferred are kits for the detection of nucleic acid from or associated with HYV. Such kits may comprise nucleic acid primers for the amplification of HYV sequences such as the primers in SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO:6 and optionally a suitable probe to detect the amplificated product.

Another preferred embodiment of the present invention to an assay kit for the detection of antibodies to hepatitis Y virus, the kit comprising at least one peptide according to the present invention and means for detecting whether the peptide is bound to antibodies to hepatitis Y virus.

A test kit for carrying out a sandwich reaction for the detection of antibodies to hepatitis Y virus may comprise, for example, a peptide according to the invention coated to a solid support, for example the inner wall of a microtest well, and either a labeled peptide according to the invention or a labeled anti-antibody.

For carrying out a competition reaction, the test kit may comprise a peptide according to the invention coated to a solid support, and a labeled specific binding molecule according to the invention.

The invention thus provides for a method for the detection of hepatitis Y virus in a sample comprising the steps of isolating nucleic acid from said sample and hybridising said nucleic acid with a nucleotide sequence derived from the genome of hepatitis Y virus.

The invention also provides for diagnosing infection with hepatitis Y virus comprising the steps of providing a sample from a patient or animal suspected of being infected with Hepatitis Y virus, providing a hepatitis Y virus or a polypeptide derived from hepatitis Y virus or functional equivalent thereof and establishing immunoreactivity of said virus or polypeptide with antibodies in said sample.

The invention also provides a method for diagnosing infection with hepatitis Y virus comprising the steps of providing a sample from a patient or animal suspected of being infected with Hepatitis Y virus, providing an antibody against hepatitis Y virus derivable polypeptides and establishing whether immunoreactive components are present in said sample.

<u>Vaccines against HYV</u>

Immunogenic preparations according to the invention can also be used to produce vaccines against infection with HYV and the resulting disease. Antibodies reactive with these

immunogenic preparations can be selected for their neutralising capacity in the infectivity assay using the in vitro culture of the "C-cell line" (ECACC deposit number 98121503)

These antibodies can be used as passive prophylaxis or therapeutic preparation aiding in removing HYV from the infected host. The peptides according to the present invention can also be used in a vaccine for the treatment of HYV infection.

In addition to an immunogenically effective amount of the active peptide the vaccine may contain a pharmaceutically acceptable excipient.

The immunogenicity of the peptides of the invention, especially the oligopeptides, can be enhanced by cross-linking or by coupling to an immunogenic carrier molecule (i.e. a macromolecule having the property of independently eliciting an immunological response in a patient, to which the peptides of the invention can be covalently linked) or if part of a protein.

Covalent coupling to the carrier molecule can be carried out using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the peptides of the invention can be coupled, e.g. using water soluble carbodiimides such as dicyclohexylcarbodiimide, or glutaraldehyde.

Coupling agents such as these can also be used to cross-link the peptides to themselves without the use of a separate carrier molecule. Such cross-linking into polypeptides or peptide aggregates can also increase immunogenicity.

Examples of pharmaceutically acceptable excipients useful in the present invention include stabilizers such as SPGA, carbohydrates (e.g. mannose, sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide, oil-emulsions (e.g. of Bayol F® or Marcol 52®), saponins or vitamin-E solubilisate.

The vaccine according to the present invention can be given inter alia intravenously, intraperitoneally, intranasally, intradermally, subcutaneously or intramuscularly.

The useful effective amount to be administered will vary depending on the age and weight of the patient and mode of administration of the vaccine.

The vaccine can be employed to specifically obtain a T cell response, but it is also possible that a B cell response is elicited after vaccination. If so, the B cell response leads to the formation of antibodies against the peptide of the vaccine, which antibodies will be directed to the source of the antigen production, i.e. the viral particle or cells expressing viral antigens on their surface. This is an advantageous feature, because in this way the cells are combatted by responses of both immunological systems.

Both immunological systems will even be more effectively triggered when the vaccine comprises the peptides as presented in an MHC molecule by an antigen presenting cell (APC). Antigen presentation can be achieved by using monocytes, macrophages, interdigitating cells, Langerhans cells and especially dendritic cells, loaded with one of the peptides of the invention or loading with protein including peptide or manosylated protein. Loading of the APC's can be accomplished by bringing the peptides of the invention into or in the neighbourhood of the APC, but it is more preferable to let the APC process the complete antigen. In this way a presentation is achieved which mimicks the *in vivo* situation the most realistic. Furthermore the MHC used by the cell is of the type which is suited to present the epitope.

An overall advantage of using APC's for the presentation of the epitopes is the choice of APC cell that is used in this respect. It is known from different types of APC's that there are stimulating APC's and inhibiting APC's.

Preferred are the listed cell types, which are so-called 'professional' antigen presenting cells, characterized in that they have co-stimulating molecules, which have an important function in the process of antigen presentation. Such co-stimulating molecules are, for example, B7, CD25, CD40, CD70, CTLA-4 or heat stable antigen (Schwartz, 1992, *Cell* 71, 1065-1068).

Fibroblasts, which have also been shown to be able to act as an antigen presenting cell, lack these co-stimulating molecules.

Instead of a vaccine with cells, which next to the desired expression products, also harbour many elements which are also expressed and which can negatively affect the desired immunogenic reaction of the cell, it is also possible that a vaccine is composed with liposomes which expose MHC molecules loaded with peptides, and which, for instance, are filled with lymphokines. Such liposomes will trigger a immunologic T cell reaction.

By presenting the peptide in the same way as it is also presented in vivo an enhanced T cell response will be evoked. Furthermore, by the natural adjuvant working of the, relatively large, antigen presenting cells also a B cell response is triggered. This B cell response will a.o. lead to the formation of antibodies directed to the peptide-MHC complex. This complex is especially found in tumor cells, where it has been shown that in the patient epitopes are presented naturally, which are thus able to elicit a T cell response. It is this naturally occurring phenomenon which is enlarged by the vaccination of APC's already presenting the peptides of the invention. By enlarging not only an enlarged T cell response will be evoked, but also a B cell response which leads to antibodies directed to the MHC-peptide complex will be initiated.

The vaccines according to the invention can be enriched by numerous compounds which have an enhancing effect on the initiation and the maintenance of both the T cell and the B cell response after vaccination.

In this way addition of cytokines to the vaccine will enhance the T cell response. Suitable cytokines are for instance interleukins, such as IL-2, IL-4, IL-7, or IL-12, GM-CSF, RANTES, MIP-α, tumor necrosis factor and interferons, such as IFN- or the chemokines.

In a similar way antibodies against T cell surface antigens, such as CD2, CD3, CD27 and CD28 will enhance the immunogenic reaction.

Also the addition of helper epitopes to stimulate $CD4^+$ helper cells or $CD8^+$ killer cells augments the immunogenic reaction. Alternatively also helper epitopes from other antigens can be used, for instance from heat shock derived proteins or cholera toxin.

Another type of vaccination having a similar effect is the vaccination with pure DNA, for instance the DNA of a vector or a vector virus having the DNA sequence encoding the peptides according the present invention (both homologues and heterologues (chimeric

protein) or repetitive). Once injected the virus will infect or the DNA will be transformed to cells which express the antigen or the peptide(s).

The invention thus provides a vaccine composition comprising a polypeptide derivable from hepatitis Y virus in substantially isolated form mixed with a pharmaceutically acceptable excipient

The invention also provides for a vaccine composition comprising a nucleic acid sequence derivable from hepatitis Y virus in substantially isolated form mixed with a pharmaceutically acceptable excipient

In Vitro culturing of HYV

The infectivity assay described in Example 5 can also be used to test antiviral substances such as nucleoside-analogues, enzyme inhibitors, agents inhibiting attachment and endocytosis of HYV to and into the host cell. The use of the assay for this purpose is also part of the invention (see Example 5).

The in vitro infection system in the "C-cell line" can be infected by HYV as shown in the examples and clones can be selected in which the HYV infectious particle is constitutively produced without causing disruption of the cell culture. The infection can be followed by several methods described herein, including performing nested RT-PCR with primers derived from SEQ ID-1or other sequences produced according to the invention and also including immunostaining with monoclonal antibody HCV-OT1F.

HYV infectious particles and polypeptides and polynucleotides produced in this way are also part of the invention.

The invention thus provides for a method for growing Hepatitis Y virus (HYV) comprising providing cells infected with HYV and propagating said cells in vitro, wherein HYV is characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement.

Anti-viral strategies

Analysis of the polypeptide and polynucleotide structure of HYV allows the identification of structural and functional targets for antiviral strategies, including nucleoside analogues, enzyme inhibitors, structural mimicry molecules and other natural or non-natural substances that can prevent or cure infection with HYV or the resulting disease. The in vitro infectivity assay can be used to monitor the effectiveness of such substances

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FIGURES

Figure 1. Recurrence of anti-HCV negative, serum HCV-RNA negative and liver HCV.OT 1F immunoreactive chronic aggressive hepatitis in a patient who underwent OLT for anti-HCV negative, serum HCV-RNA negative and HCV.OT 1F immunoreactive associated terminal liver cirrhosis.

Figure 2 : Serum aminotransferases (AST and ALT) and liver HCV.OT 1F immunoreactivity before and after inoculation (time 0) of chimpanzee Sylvia.

Figure 3 : Serum aminotransferases (AST and ALT) and liver HCV.OT 1F immunoreactivity before and after inoculation (time 0) of rhesus monkey I FU.

Figure 4 : Aminotransferase (ALT) values before and after inoculation of rhesus monkey 8925.

Figure 5 Cesium chloride gradient centrifugation of plasma of patient OHN

Figure 6 Cesium chloride gradient centrifugation of supernatant fraction S3

EXAMPLES

EXAMPLE 1

CLINICAL CHARACTERISTICS OF PATIENTS WITH HEPATITIS Y

Anti-HCV negative, HCV-RNA negative chronic non B-hepatitis associated with immuno-histochemical reactivity for a monoclonal antibody HCV.OT 1F

Previously we have reported the successful detection of hepatitis C virus core antigen by a monoclonal antibody HCV.OT 1F (, developed against a peptide with the sequence RTQQRKTKRSTNRRR (according to EP 0537856) which has 8 of 15 aminoacids in common with the sequence of the HCV core conserved region (according to EP 0388232)) in liver biopsy specimens of patients with chronic hepatitis C, who were positive for anti-HCV and serum HCV-RNA (Yap SH et al., 1994. J Hepatol: 20 ; 275-81). The cytoplasmic granular immunoreactivity was absent in the cytoplasm of hepatocytes of patients suffering from various liver diseases, who were not suspected for infection with non-A, non-B agents. However, the typical immunoreactivity was also detected in the liver cells of a limited number of patients with chronic hepatitis B, suspected for infection with non-A, non-B agents, despite there was a lack of anti-HCV or serum HCV-RNA.

This striking finding can be considered as false positivity but it can also indicate that the HCV.OT 1F-staining is more sensitive for detection of HCV-infection than the classical assays. In addition, the finding may also be the result of HCV.OT 1F cross-reactivity to a unique agent associated with chronic non C-hepatitis. In an attempt to identify additional cases with chronic hepatitis and lacking of anti-HCV and serum HCV-RNA, but HCV.OT 1F immunostaining reactive in liverbiopsies, we initiated a prospective study in patients who were admitted to our liver unit for a liver biopsy because of acute or chronic liver disease. These patients underwent hepatological work-up including determination of serological markers of known hepatitis virus infection, autoantibodies for the diagnosis of autoimmune liver disease and tests for metabolic liver disease, such as haemochromatosis and Wilson's disease. Data on anti-HCV and serum HCV-RNA were also collected from the past examinations and during follow-up in order to exclude intermittent viremia which may exist in seronegative chronic hepatitis C patients. In addition, a possible infection with HGV was also

investigated by detection of serum HGV-RNA using PCR-assay. Liver biopsies were reviewed for histology and examined for granular immunoreactivity to the monoclonal antibody HCV.OT 1F.

From the 165 consecutive patients investigated so far, fourty-two patients (25.5 %) were negative for anti-HCV, serum HCV-RNA and for liver HCV.OT 1F immunoreactivity (group A). Twenty-four patients (14.5 %) were positive for anti-HCV, serum HCV-RNA and for HCV.OT 1F immuno-staining (group B). Ninety-three patients (56.3 %) were liver HCV.OT 1F immunoreactive but lacking of anti-HCV and serum HCV-RNA (group C). In addition, a limited number of patients had a inconsistentpattern of anti-HCV, serum HCV-RNA and HCV.OT 1F immunoreactivity in the liver biopsies (table 1).

Table 1. The results of a prospective study for liver HCV.OT 1F immunoreactivity in 165 consecutive patients who underwent a liver biopsy because of acute or chronic liver disease and the relation with HCV serological markers.

	Number of patients	Anti-HCV	Serum HCV-RNA	Liver HCV.OT 1F immunoreactivity
Group A	42	negative	negative	negative
Group B	24	positive	positive	positive
Group C	93	negative	negative	positive
	2	negative	positive	positive
	2	positive	negative	positive
	1	positive	negative	negative
	1	positive	positive	negative

Most of these patients (> 95 %) underwent a liver biopsy because of elevated serum liver enzymes. Some patients had a biopsy for diagnosis of haemochromatosis or for the existence of a malignant tumor. In addition, a limited number of liver specimens were recruited for investigation from post-mortem donor livers, which were for some reasons not suitable for transplantation.

The elevated serum liver enzymes in patients from group A could be attributed to various liver disorders, as shown in table 2. The prevalence of hepatitis B infection in this group of patients was 12 %, and an association with suspected excessive alcohol intake was found in 12 % of the cases.

Table 2. Histological findings of liver biopsies of patients from group A (anti-HCV negative, serum HCV-RNA-negative and HCV.OT 1F immuno-non-reactive patients; total number : 42).

Drug-induced hepatitis (5)
Donorliver, no signs of hepatitis (8)
Cholangiocarcinoma (1)
Hepatitis B virus infection (1 acute, 2 chronic aggressive hepatitis, 2 active cirrhosis)
Ito-cell hyperplasia (vit. A related) (1)
Steatosis (2)
Cryptogenic cirrhosis (1)
Necrotizing hepatitis (4)
Autoimmune chronic hepatitis (1)
Adenocarcinoma (1)
Vanishing bile duct disease (1)
PBC (2)
Primary haemochromatosis (2)
Chronic active hepatitis (1)
Minimal hepatitis (2)
Liver metastasis (1)
Granulomatous hepatitis (1)
Caroli disease (1)
Nodular regenerative hyperplasia(1)
Normal liver parenchyma next to presumed focal lesion (1)

All patients from group B with diagnosis of chronic hepatitis C had elevated serum liver enzymes. More than 90 % of the patients from group C had also abnormal serum liver enzymes at least two weeks before the liver biopsy was taken. The histological findings of the liver biopsies of patients from group C are shown in table 3. More than 75 % of the patients had a variable degree of portal and/or periportal inflammation. The association with hepatitis B virus infection was 11,8 %, which was comparable with the findings in group A. Thirty-one

patients were tested for the presence of serum HGV-RNA and only one was found to be positive. There was a suspicion of excessive alcohol intake in 27 patients (28 % in group C, as compared to the 12 % in group A. However, the degree of portal and periportal inflammation present in these liver biopsies could not be attributed solely to alcohol induced liver injury. It was suspected that additional viral infection mightplay a role.

Table 3. Histological findings of patients from group C (anti-HCV negative, serum HCV RNA negative and liver 1F2-immunoreactive patients; total number : 93).

Portal and/or periportal inflammatory infiltrates (29)
Intralobular inflammatory infiltrates (6)
Steato-hepatitis (15)
Steatosis (4)
Cirrhosis, hepatocellular carcinoma and portal inflammatory infiltrates (2)
Active cirrhosis and hepatitis B virus infection (3)
Chronic hepatitis B virus infection (7)
Chronic hepatitis B virus infection and steatohepatitis (1)
Inactive cirrhosis (4)
Primary biliary cirrhosis (1)
Ductopenia (1)
Cirrhosis and steatohepatitis (9)
Metastasis of a neuroendocrine tumor (1)
Primary hemochromatosis and portal inflammatory infiltrates (3)
Cirrhosis and hepatitis G virus infection (1)
No obvious abnormalities (6)

It is also interesting to note that 5 patients from group C had extrahepatic pathology : polymyositis (in one patient), periarteritis nodosa (1), mixed connective tissue disease (1), extrinsic allergic alveolitis (1) and one patient with an acute episode of fever, arthralgias, urticaria and eosinophilia.

Thirteen patients in group C had a follow-up of more than 3 years. Seven showed a persistent rise in serum liver enzymes, while 6 had a transient pattern. Seven out of ten patients with a follow-up of 1 to 3 years had persistent elevated serum liver enzymes and 3 had a transient rise. Normalisation of serum ALT was noted in one patient, who remained liver HCV.OT 1F immuno-reactive when he underwent a new liver biopsy because of the development of hepatocellular carcinoma.

From this prospective study of 165 patients who were admitted to the liver unit for liver biopsy because of acute or chronic liver disease we have identified a group of 93 patients with elevated serum liver enzymes and associated with histological findings of portal and/or periportal inflammation and a granular cytoplasmatic staining with monoclonal antibody HCV.OT 1FThese patients however, repeatedly showed the absence of anti-HCV and serum HCV-RNA.

Previously, we have described a similar finding but in a limited number of patients in a previous report (Yap SH et al., 1994 see above). All patients had concomittant hepatitis B virus infection, but were also suspected for a coinfection with non A, non B agents. In the present study of 93 patients the prevalence of HBV infection was 12 % which was comparable to the figure found in group A patients who were negative for anti-HCV and serum HCV-RNA and liver HCV.OT 1F immuno-non-reactive. Nevertheless, there is an association with a history of excessive alcohol intake in group C patients as compared to the group A. The liver histology of these patients showed however, pronounced portal and/or periportal inflammation which can not be attributed to alcohol intake. The liver histology is in fact compatible with the findings in chronic hepatitis C patients. Another particular feature of these patients is the frequent finding (29 patients) of steatosis or steatohepatitis.

From this study, we may therefore conclude that apart from HCV infection, monoclonal antibody HCV.OT 1F has identified a uniqueform of hepatitisnon -A to G, which may be caused by a transmissible agent.

Laboratory procedures

Assay for liver enzymes

Serum aminotransferases (sALT and sAST), alkaline phosphatases and gamma-glutamyl transferases were assessed using routine laboratory procedures.

Assay for anti-HCV antibodies

HCV antibodies were detected by a second generation anti-HCV enzyme-linked immunosorbent assay from Abbott (MEIA, AxSYM) and Sanofi (Monolisa). In some patients the Chiron RIBA HCV 3.0 SIA was used as a confirmatory test.

RNA extraction. RNA was extracted from 200 µl serum or EDTA treated plasma, in a single step acid guanidinium thiocyanate-phenol-chloroform extraction procedure, as described by Chomczynski & Sacchi (1987). After isopropanol precipitation, the RNA was dissolved in 8 µl DEPC-treated water.

HGV- and HCV-RNA detection by RT-PCR assay. For reverse transcription, the RNA was denatured by incubation for 10 min at 60°C. To the RNA fraction, 12 µl reverse-transcription mix was added, containing 200 units Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Gibco BRL, Bethesda, MD), 30 units placental RNase inhibitor (Pharmacia, Uppsala, Sweden), 80 pmol random primers (Boehringer Mannheim, Germany), 0.5 mM of each deoxynucleotide triphosphate (dNTP; Pharmacia), 10 mM dithiotreitol, an appropriate volume of 5 x reverse transcriptase buffer, and DEPC treated water. The cDNA synthesis was carried out at 37°C for 1 hour and stopped by heating in boiling water for 1 minute. Subsequently, single round, double round nested or semi-nested PCR was performed. Briefly, the first PCR mixture contained 5 µl cDNA solution, 20 pmol (HGV) or 50 pmol (HCV) of each primer, 0.2 mM of each dNTP and 1 unit Primezyme DNA polymerase (Biometra, Göttingen, Germany) in a total volume of 50 µl PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin). Samples were overlaid with mineral oil and subjected to thermal cycling. When nested or semi-nested PCR was performed, 3 µl of first round PCR product was added to a new PCR mixture containing the appropriate nested or semi-nested PCR primers for a second amplification. Amplified DNA was visualized by agarose-gel electrophoresis (2%) and ethidium bromide staining.

HGV-RNA was detected by single round PCR with primers specific to the non-structural (NS) 3 region (HGV-NS3.S : 5'GGTGAGATTCCCTTCTATGGGCATGG; HGV-NS3.A : 5'CCTCAGCAGTAGTGGAACAGGATTCGG) or by semi-nested PCR with primers for the 5' non-coding region (NCR) (HGV-NCR.S : 5'CGGCCAAAAGGTGGTGGATG; HGV-NCR.A1: 5'GGTTTAACGACGAGCCTGACG; HGV-NCR.A2: 5'CGGTAGG-GCCAACACCTGTGG where A1 is the anti-sense primer for the first round and A2 for the second round amplification) or the NS5 region (HGV-NS5.S: 5'CTCTTTGTGGTAGTAGCCGAGAGAT; HGV-NS5.A1: 5'TGAGTCAGAGGACGGGGTATCC;. HGV-NS5. A2: 5'ATCTGAGCTGCTCTCGGTAACCG where A1 is the anti-sense primer for the first round and A2 for the second round amplification). Cycling conditions were 1 min 95°C, 1 min 55°C, 1 min 72°C. For single round PCR 45 cycles were

performed, for semi-nested PCR, 45 and 30 cycles were performed for the first and second round amplification respectively.

HCV-RNA was detected by nested PCR with primers for the 5'NCR (first round: HCV-NCR.S1 : 5'CCACCATAGATCTCTCCCCTGT; HCV-NCR.A1 : 5'ATACTCGA GGTGCACGGTCTACGAGACCT; second round : HCV-NCR.S2 : 5'AGATCTTCA-CGCAGAAAGCGT; HCV-NCR.A2: 5'CACTCTCGAGCACCCTATCA GGCAGT). First round cycling conditions were 96°C for 30 seconds, 48°C for 45 seconds, 72°C for 1 minute, 35 cycles. Second round cycling conditions were 96°C for 30 seconds, 42°C for 45 seconds, 72°C for 1 minute, 30 cycles.

Immuno-histochemistry

Immuno-histochemistry was performed as described previously (Yap SH et al., 1994, see above) on 4-µm-thick cryostat sections of fresh frozen materials using a three-step indirect immuno-peroxidase procedure. Sections were incubated overnight at 4°C with the monoclonal antibody HCV.OT 1F (purified IgG1 : 20 ng/µl). The secondary and tertiary antibodies consisted of peroxidase-conjugated rabbit anti-mouse and peroxidase-conjugated swine anti-rabbit IgG, respectively (both obtained from Dakopats a/s Copenhagen, Denmark; working dilution 1/50 and 1/100, respectively). Each incubation was performed for 30 minutes at room temperature and followed by a wash in three changes of phosphate buffered saline, pH 7.2. The reaction product was developed by incubation for 15 min in 100 mM acetate buffer (pH 5.2), containing 0.05 % 3-amino-9-ethyl-carbazole and 0.01 % H_2O_2, resulting in bright red staining of immuno-reactive sites.

In order to abolish a weak "non-specific" background reactivity, the monoclonal antibody was pre-incubated overnight with the homogenate of normal human liver tissue before use.

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EXAMPLE 2

Clinical evidence for the existence of a transmissible agent causing non-A-G hepatitis associated with HCV.OT 1F-liver immunoreactivity

In order to demonstrate that non-B, non-C chronic hepatitis associated with HCV.OT 1F liver immunoreactivity is caused by a a transmissible agent, we investigated patients who underwent an orthotopic liver transplantation. The setting of an orthotopic liver transplantation

(OLT) may represent an excellent in vivo model for demonstration of the transmissibility of a putative infectious agent causing chronic hepatitis.

For this study six patients (1-3, 6-8) who were HCV.OT 1F liver immunoreactive and underwent OLT were included. One patient was negative for HCV.OT1F immunostaining and in 2 patients the immunostaining was not performed before OLT. .

The serological markers of HCV infection and HCV.OT 1F liver immunoreactivity of these patients are shown in table 4.

Table 4. Serological markers of HCV-infection and liver HCV.OT 1F immunoreactivity in patients who underwent an orthotopic livertransplantation.

Patient	Indication for OLT	Markers for HCV infection			Liver histological findings after OLT
		Before OLT	*Liver donor*	*After OLT*	
1	Cirrhosis Terminal liver disease	anti-HCV + HCV-RNA + HCV.OT 1F +	anti-HCV - HCV-RNA - HCV.OT 1F -	anti-HCV + HCV-RNA + HCV.OT 1F +	Persisting hepatitis at 2 months
2	Cirrhosis + hepatocellular carcinoma	anti-HCV + HCV-RNA + HCV.OT 1F +	anti-HCV -	anti-HCV + HCV-RNA + HCV.OT 1F +	Persisting hepatitis at 6 and 9 months
3	Cirrhosis Terminal liver disease	anti-HCV + HCV-RNA	anti-HCV -	anti-HCV + HCV-RNA + HCV.OT	Cellular rejection at 1 week

		+ HCV.OT 1F +		1F +	
4	Cirrhosis Terminal liver disease	anti-HCV - HCV-RNA -	not known	anti-HCV + HCV-RNA + HCV.OT 1F +	Persisting hepatitis for 7 years
5	Cirrhosis Terminal liver disease	anti-HCV - HCV-RNA -	not known	anti-HCV + HCV-RNA + HCV.OT 1F +	Persisting hepatitis for 7 years
6	Cirrhosis Terminal liver disease	anti-HCV - HCV-RNA - HCV.OT 1F +	anti-HCV -	anti-HCV - HCV-RNA - HCV.OT 1F +	Persisting hepatitis for 3 years
7	Primary Biliary Cirrhosis Terminal liver disease	anti-HCV - HCV-RNA - HCV.OT 1F +	anti-HCV - HCV-RNA - HCV.OT 1F -	anti-HCV - HCV-RNA - HCV.OT 1F +	Persisting hepatitis at 3 and 12 months
8	Fulminant liver failure	anti-HCV - HCV-RNA - HCV.OT 1F +	anti-HCV -	anti-HCV - HCV-RNA- HCV.OT 1F +	Transient hepatitis at 2 months; normal biopsy at 1 year (and 1 F-2 negative)
9	Primary Biliary Cirrhosis Terminal liver disease	anti-HCV - HCV-RNA - HCV.OT 1F -	anti-HCV - HCV-RNA - HCV.OT 1F -	anti-HCV - HCV-RNA- HCV.OT 1F +	Persisting hepatitis at 1 year

Patients 1-5 were anti-HCV and serum HCV-RNA positive after OLT. All the patients in this group developed biochemical and histological changes of chronic hepatitis after OLT. Patient 1-3 had a HCV reinfection. These patients were positive for anti-HCV, serum HCV-RNA and HCV.OT 1F immunoreactivity before OLT. The liver donors of these patients were anti-HCV negative. In addition, the donor liver of the first patient before OLT was shown to be HCV-RNA negative and HCV.OT 1F immuno-non-reactive.

Case 4 and 5 were anti-HCV and serum HCV-RNA negative before OLT. These patients were infected by HCV during or some time after OLT.

Patient 6-8 were negative for anti-HCV and for serum HCV-RNA but HCV.OT 1F liver immunoreactive before OLT. Despite the anti-HCV and serum HCV-RNA remained negative, the liver grafts became HCV.OT 1F immunoreactive at some time after OLT and remained immunoreactive during follow-up. Patient 6 developed a chronic aggressive hepatitis (figure 1). In patient 7 only a mild persisting hepatitis was noted. Patient 8 was transplanted for fulminant liver failure associated with HCV.OT 1F liver immunoreactivity and the transplanted liver became HCV.OT 1F positive 2 months after OLT. One year after OLT the liver enzymes of this patient were normalized, and no HCV.OT 1F immunoreactivity could be observed in the liver biopsy specimen.

Patient 9 was negative for anti-HCV, serum HCV RNA and liver HCV.OT 1F immuno-non-reactive before OLT and developed a HCV.OT 1F positive chronic persisting hepatitis, with fluctuating liver enzymes after OLT. She remained anti-HCV and HCV-RNA negative for 1 year after OLT.

All liver donors of the patients 6-9 were anti-HCV negative. In addition, the liver biopsy specimens of patient 7 and 9 two hours after reperfusion of the new grafts were HCV-RNA negative, and HCV.OT 1F immuno-non-reactive. No specimens of donor livers were available for investigation from patient 6 and 8.

For patients who underwent an OLT as shown in this study, HCV.OT 1F immunostaining in liver biopsies could be a sensitive marker for reinfection (patient 1-3) or *de novo* infection (patient 4-5) of hepatitis C virus. These findings are in good agreement with the

previously reported good sensitivity and specificity of HCV.OT 1F immunostaining in patients with HCV infection (Yap S.H. et al., 1994. J Hepatol; 20 :275-81).

However, in this study we identified 3 patients (patient 6-8) with a persisting hepatitis after OLT who were liver HCV.OT 1F immunoreactive, but remained serum HCV-RNA and anti-HCV negative during follow-up. A review of the viral and histological data of these patients before OLT, showed that HCV.OT 1F liver immunoreactivity was found prior to OLT, despite anti-HCV and serum HCV-RNA were negative and remained negative after OLT. Finally, in one patient (patient 9), HCV.OT 1F immunoreactivity associated with chronic hepatitis was found after OLT while it was negative before OLT. This finding is strongly suggestive for a *de novo* infection.

From these findings based on clinical studies of patients who underwent OLT, we can therefore conclude that anti-HCV negative, serum HCV RNA negative and liver HCV.OT 1F immunoreactivity associated liver disease is caused by a transmissible agent.

EXAMPLE 3

REACTIVITY PATTERN OF MONOCLONAL ANTIBODY HCV.OT 1F

A total of 4550 random linear dodecapeptides (12-mers), synthesized in credit-card format minipepscan cards, were screened with mAb HCV.OT 1F (1/100) as described previously (Slootstra et al., 1995, Molecular Diversity 1, 87-96)

1. Summary of Results

The results are given in Table 1 below. The results are ranked for Optical Density (OD) values and the actual OD value found is followed by the sequences of the peptide in the respective test well.

Alignment top 25 peptides

The consensus tripeptide CXR, with CHR and CNR in particular, is a dominant sequence motif in the top 25 peptides

Analysis of single amino acids

In the top 200 peptides the frequency of the amino acids C and R is significantly higher compared to that of the other 18 amino acids

Analysis of dipeptide motifs

The highest scoring dipeptide motifs contain the amino acids C and/or R. Flanking high scoring amino acids include A, F, K, M and Q.

Analysis of tripeptide motifs

The occurrence of all possible 8000 tripeptides (000) as well as all possible spaced tripeptides (OXXXXOXO, OXOXO etc. etc.) in the top 200 peptides was determined. Most of the top tripeptides contain the sequence motif CXR. Flanking high scoring amino acids include A, F and M).

Positional analysis of CXR motif

The sequence motif CXR has a preferred location in the second half of the peptide sequence.

2. Conclusions

The various analyses revealed that CHR or CNR plus the flanking residues A, F, K, M and Q are involved in antibody binding. We propose the following short sequences as candidate energic cores of a mimotope: ACHR, ACNR, FCHR, FCNR, CHRF, CNRF, CHRXA, CNRXA, MXCHR, MXCNR, FXCHR, FXCNR (X, various residues). We expect that these motifs form a firm basis for the development of useful mimotopes.

Another important conclusion is that none of the most reactive peptides has a significant homology to the peptide the monoclonal antibody HCV.OT 1 F was raised against (RTQQRKTKRSTNRRR), except for the relatively high frequency of occurrence of the aminoacid Arginine (R).

TABLE 5

TOP 100 REACTIVE 12-MER PEPTIDES FROM A 4550 RANDOM PEPTIDE LIBRARY, TESTED WITH MONOCLONAL ANTIBODY HCV.OT.1F

Peptides are ranked based on Optical density. The actual OD value is given followed by the sequence of the peptide in the respective test well.

2919	TINTETCARAAV
2676	MILRACHRQLCV
2574	RNMSKTSASAVE
2557	EEKKQCNFTKLD
2461	DMCKTIWACRRI
2431	RHYYVLCNRGTA
2410	CYCWRCARVMFN
2313	QIMAEPRQTCNY
1989	SEWAFCMRFIHQ
1677	YRVQHCRRQRDT
1652	HTSFSCMRDVFM
1502	CNRPMYFKIPYI
1450	TERSFWVMACHR
1435	QCHRVAYIGGCN
1420	IVYLKDICKRRQ
1381	RSRADQSSQKAC
1377	ASILCLRISHQT
1377	KVLIVSCNRHKT
1335	WMVACHLRLMSD
1246	FPCLMLICKRFF
1225	QSQVHMIEKNRD
1206	AGASDKRLCNKI
1151	GTNHRNCWRLDQ
1143	IFVQVQFACNIN
1077	VEKTAFNCNVMI

1073	RYQWNLPCNIMR
1062	NPICQRFCATWK
1049	SNDGCQFDILPK
1038	TWGPSNQKTSKL
1018	MKMIWRDVQCNK
956	ALELYAMRQCFC
944	YCCRLAHQNCKF
937	LMMHIVLWLCNK
922	LQCFICRQSPAN
919	TMFCLRRSCTRS
886	HDEACCRFFTHE
876	TGQNLRHCRAMC
870	AGYACKFSREGE
868	IRCDIIYCWRQC
842	GDDWSHGCLRVF
823	MEQAVQIHCFRQ
819	GDEWTQPRFCQR
791	EPEKNIERCAIA
791	AAMTFYCHRTAF
789	DNLLWEVCDRYM
785	KWNGYHEMPCNQ
759	DYYTMNCERKFN
758	RGLRWIFRRCFR
756	TEWVCFRRWPTA
739	QELHEPIKCIRL
738	CSDVVETRCQID
738	QQYSCSCNTQLD
722	GVFGICHFEKMA
721	KRMCKNMQECGK
716	SFMCRWDISDRQ
712	QQRWCLRTVMEY
696	CFCAMVIRSKKA

691 VWALRCSRIRDH
691 ACKFCQNGQSQM
687 GMELNWDHHKQR
681 ENFFVVGETVAC
681 QTMGGPSCQGIM
676 KGAMMRVRWTAC
676 TRHGRHVPTRRC
674 WDFWMQKEKGKI
671 RCMYDVAYVTCI
671 LMGNKMRCRMDR
665 WKTAYMPPNRLD
662 WHETCMYQVFNQ
661 SQRMWKENVMAT
651 CCRDRIKVEDYA
651 CACTIHWCFCCR
648 HPQGATNKMEGF
645 CGLSNCDQTCHR
645 IHKMTFGQMCLN
636 EQPWKNQDFKLS
634 WEAVVGFCARKI
628 ITWQWKNRAVFS
628 LRVCHAPVSFMC
627 PWPFCHIHAEFA
627 FKCNMMEQNHVI
625 YDWWQHNLTSDV
625 QHTKASWLQRTC
622 MKVRCVDWRVVA
620 HQFIARWQVWER
620 MKGMYTDDAILF
620 YMLKQKCSEQIC
619 YFCADYIMQNSA
614 CDYWGVFWKYNI

614	YCNTLPDDFITK
614	DIRQNWTMWCSR
610	CPQVTESMHQLD
610	HEQRRCKIVHKI
610	PYGNRVSNLTSL
608	KVWGIPFWVNAM
603	QKCAYGFLILTP
603	NCTEGHAVILMQ
602	HFQGHQGVNWNY
602	PRHKDVECMSGQ
602	QCAKYADLCYLT

EXAMPLE 4

Transmission studies in primates

1. Introduction

From previous clinical studies of patients who underwent orthotopic liver transplantation, we demonstrated that anti-HCV negative, serum HCV RNA negative and liver HCV.OT 1F immunoreactivity associated liver disease is most likely caused by a transmissible agent. In order to determine whether this type of liver disease can also be transmitted from human to animals, we initiated transmission studies in primates. Additional objectives of the current project were (1) To characterize the symptoms of this form of hepatitis in primates by studying clinical, biochemical, histopathological and virological parameters; (2) To obtain a sufficient amount of PBMC, liver and lymphoid tissue for laboratory investigations, aimed at characterization of the infectious agent.

2. Materials and methods

2.1 Animals

Animals for the transmission studies were secured through the Biomedical Primate Research Center (BPRC) in Rijswijk, the Netherlands. Animal care and experimental conditions were in accordance with protocols that met all relevant requirements for the human care and the ethical use of primates in an approved facility. One female chimpanzee *(Pan troglodytes)* and four rhesus monkeys *(Macaca mulatta),* which were selected for the study, met the following criteria: *(1)* They had not been inoculated before with human serum or serum derived products. (2) They were negative for antibodies against hepatitis C virus and the core protein of hepatitis B virus. Hepatitis C and G virus RNA could not be detected and the tuberculin reaction was negative. (3) They showed normal levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase, and lactate dehydrogenase (LDH), which were determined at least 8 times in a 4-8 weeks interval before the inoculation.

(4) All animals showed normal liver histology with the absence of liver immunoreactivity using the monoclonal antibody HCV.OT 1F.

2.2 Transmission studies

2.2.1 Primary inoculations

Four different inocula were used in the first transmission study and were derived from two patients suspected of infection with a non A-G hepatitis inducing agent. The first patient (OHN) is a woman of 35 years old with a history of a blood transfusion in 1983. In august 1995 she complained of tiredness and was found to have a dramatic rise in serum ALT (8-10 times the upper normal limit), persisting for six months at the time of the liver biopsy. The liver biopsy showed a marked steato-hepatitis, with a granular staining for the monoclonal antibody HCV.OT 1F. Anti-HCV, serum HCV-RNA and HGV-RNA, and other known viral and autoimmune markers were repeatedly negative. There was no evidence for metabolic liver pathology. Plasma and peripheral blood mononuclear cells (PBMC) were with her permission stored at -80°C for further study. The second patient (CD) is a man of 39 years old who presented with recurrent oesophageal variceal bleeding as a complication of cirrhosis in December 1995. The cirrhosis could not be attributed to any known viral, toxic or metabolic cause, except for a positive immunoreactivity to HCV.OT 1F. In march 1996 he underwent an orthotopic liver transplantation. The explanted liver showed a micronodular type of cirrhosis,

with a pronounced mononuclear cell infiltrate in the portal tracts and a positive immunoreactivity using the monoclonal antibody HCV.OT 1F The left liver lobe of the explant was used for isolation of hepatocytes by a two-step perfusion technique as described previously (Moshage H et al., 1988). The isolated primary human hepatocytes were subsequently cryopreserved. In addition, pre-transplantation plasma was also stored at -80°C.

Inoculum A contained 20 million cryopreserved human hepatocytes from patient CD, 2 ml plasma from patient CD, 5 ml plasma and PBMC (out of 20 ml blood) from patient OHN. Inoculum B was similar to inoculum A, but included no PBMC. Inoculum C contained only 20 million cryopreserved hepatocytes from patient CD, while inoculum D was prepared from 5 ml plasma of patient OHN.

Inoculum A was injected intravenously to chimpanzee Sylvia. Inocula B, C and D were injected to the rhesus monkeys I FU, 8925 and IXK respectively. The rhesus monkeys were monitored clinically and biochemically at least every two weeks for six months. Liver biopsy was performed monthly. The chimpanzee was followed in a similar but extended protocol and had an additional liver biopsy 18 months after inoculation.

2.2.2 Passage of 1 FU infectivity

Rhesus monkey I FU developed hepatitis, which was associated with an impressive liver immunoreactivity for the monoclonal antibody HCV.OT 1F (as described below in 3. *results).* Serum collected during the early acute phase (from 4 to 12 weeks after inoculation) was pooled (inoculum E) and injected to rhesus monkey BB58. This primate was monitored clinically, biochemically and histologically for 6 months.

2.3 Immunohistochemistry

Immunohistochemistry was performed as described previously (Yap SH et al., 1994) on 4 µm-thick cryostat sections of fresh frozen materials using a three-step indirect immuno-peroxidase procedure.

Sections were incubated overnight at 4 ~C with the monoclonal antibody HCV.OT 1F (purified IgG 20 ng/µl). The secondary and tertiary antibodies consisted of peroxidase-conjugated rabbit anti-mouse and peroxidase -conjugated swine anti-rabbit lgG, respectively

(both obtained from Dakopats a/s Copenhagen, Denmark; working dilution 1/50 and 1/100, respectively). Each incubation was performed for 30 minutes at room temperature and followed by a wash in three changes of phosphate buffered saline, pH 7.2. The reaction product was developed by incubation for 15 minutes in 100 mM acetate buffer (pH 5.2), containing 0.05 % 3-amino-9-ethyl-carbazole and 0.01 % H_2O_2, resulting in bright red staining of immuno-reactive sites. In order to abolish a weak "non-specific" background reactivity, the monoclonal antibody was pre-incubated overnight with the homogenate of normal human liver tissue before use.

2.4 Assay for liver enzymes

Serum activities of aminotransferases (ALT and AST), alkaline phosphatase and gamma-glutamyltransferase (GGT) were determined using routine laboratory procedures. Baseline serum levels in international units per liter (IU/L) were established on serum specimens obtained weekly or bi-weekly. A minimum of eight values were obtained prior to inoculation. Cut-off values were determined for each animal, based on the mean enzyme value plus 3.75 times the standard deviation. Enzyme values above the cut-off value were interpreted as abnormal and suggestive of liver damage, as has been performed by others in similar transmission studies (Schlauder G et al., *1995).*

3. Results

3.1 Primary inoculations

3.1.1 Chimpanzee Sylvia

From week 5 to 7 after inoculation there was a transient increase in serum AST above the baseline cutoff value, and a discrete rise in serum ALT as shown in figure 2. A liver biopsy performed 3 weeks after inoculation showed an increase in mononuclear cells in the parenchyma (lobular hepatitis) which was maintained during the observation period of 6 months. In addition, there was some degree of portal and periportal inflammation at week 7,11 and 13. In addition, the biopsy taken at the time of the peak AST value (week 7) showed a moderate degree of piecemeal necrosis. This biochemical and histological hepatitis was associated with a granular immunoreactivity to HCV.OT 1F, in the form of cytoplasmatic

granules in the periphery of the liver cells which remained present up to 18 months after inoculation, with the exception of a negative staining at six months.

3.1.2 Rhesus monkey IFU

In contrast to the chimpanzee data there was no rise in serum liver enzymes following the inoculation throughout 6 months of observation (figure 3). However, the liver biopsy taken 1 month after inoculation showed a discrete lobular hepatitis with a few cells showing a positive immunoreactivity to HCV.OT 1F At week 8 there was a moderately dense infiltrate in the portal tracts and borderline interphase hepatitis, associated with an intense HCV.OT 1F immunoreactivity. The subsequent biopsies showed a virtually normal liver parenchyma and a negative HCV.OT 1F immunoreactivity, with the exception of a few positive cells in the liver specimen at 20 weeks. Unfortunately, the final liver biopsy at six months was complicated by a hemorrhage and the primate died.

3.1.3 Rhesus monkey 8925

The inoculation of 20 million putatively infected hepatocytes resulted in a rise of serum ALT above the baseline cutoff value at week 11, and from week 16 to 17 (figure 4). The first liver biopsy at 4 weeks after inoculation was normal, including a negative HCV.OT 1F immunoreactivity. However, the staining became positive on the subsequent biopsy at 8 weeks and remained weakly positive for 6 months. At 10 weeks there was a distinct lobular hepatitis, which resolved on the subsequent biopsies. Eight months after inoculation the animal was sacrificied and the hepatocytes were subsequently isolated and cryopreserved for further study. However, at that time the liver was found to have lost immunoreactivity to HCV.OT 1F.

3.1.4 Rhesus monkey IXK

Inoculation with 5 ml plasma of patient OHN did not result in biochemical or histological hepatitis and there was no immunoreactivity to HCV.OT 1F.

3.2 Passage of IFU infectivity

Rhesus monkey BB58 did not have a rise in serum aminotransferases. However, two months after inoculation a focus of necrotising hepatitis was seen in the liver specimen and at 3 months a transient typical granular immunoreactivity to HCV.OT 1F was seen in a minority of the liver cells.

4 Discussion

From this transmission studies, both in chimpanzee and rhesus monkeys, we have provided solid evidence that the HCV.OT 1F immunoreactivity is caused by a transmissible agent, present in the hepatocytes of a patient (CD), who developed cirrhosis, characterized by liver immunoreactivity to HCV.OT 1F, despite negative anti-HCV or serum HCV-RNA. Moreover, this immunoreactivity to HCV.OT 1F was associated with a transient rise in serum ALT and a persistent inflammation in the liver parenchyma (both lobular and portal) in the chimpanzee study. The rhesus monkey I FU inoculated with the mixed inoculum, derived from the liver and plasma of patient CD and plasma of patient OHN, also developed this hepatitis, which resolved after 6 months. In addition, the development of transient liver immu no-reactivity to HCV.OT 1F in rhesus monkey 8925, who was only inoculated with the hepatocytes of patient CD, further underscores the transmissibility of the putative non-A-G agent. The data of the passage study of I FU infectivity (rhesus monkey BB58) suggest that the agent can be transmitted by the serum of a primate with liver immunoreactivity to HCV.OT 1F. The lack of liver immunoreactivity in the primate IXK, who was inoculated only with the plasma of patient OHN, therefore suggests that the putative agent was not present in a sufficiently high titer in that plasma batch.

In conclusion, primary inoculation studies have shown that liver HCV.OT 1F immunoreactivity and associated hepatitis can be passed on a chimpanzee or rhesus monkeys by infusion of hepatocytes from a patient with non-A-G. In addition, liver HCV.OT 1F immunoreactivity could be transmitted from one rhesus monkey to another by infusion of serum, collected during the episode of hepatitis, which developed in a rhesus monkey after primary inoculation with a human putative non-A-G inducing agent.

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EXAMPLE 5

Characterization of an *in- vitro* assay using an immortalized human hepatocyte cell line for demonstration of hepatitis Y infection

1.Definition

In this report we summarize the characterization of an *in-vitro* assay of HY infection, which is based on the incubation of immortalized human hepatocytes with inoculum containing non-A-G transmissible agent(s) in a rotatory cell culture system.

2. Materials and methods

2.1 Development and characterization of the cell line

The immortalized human hepatocyte line C (deposited at the ECACC under accession number 98121503) has been developed in our laboratory from primary cultures of human hepatocytes as described previously (Fourneau I et al., 1997). In brief, human hepatocytes were isolated from human liver tissues, which could not be used for transplantation for some reasons. These liver tissues were obtained from post mortem liver donors with approval of the

medical Ethical Committee for human experimentations. Hepatocytes were isolated using a two-step collagenase perfusion method (Moshage H et al., 1988; Rijntjes PJM et al., 1986). Freshly isolated hepatocytes were cultured on six well plates (Nunc, Roskilde, Denmark), pretreated with human liver biomatrix (Rijntjes PJM et al., 1988) and were allowed to attach to this matrix in complete WE medium (William's E medium, (Gibco) supplemented with10% fetal calf serum (Gibco), 2mM L-Glutamine(Gibco) 20 mU/ml insulin(Novo Nordisk Pharma) 50 nm dexamethason and antibiotics).

The isolated hepatocytes were kept in culture for two months or longer. During this period of culture, cells were regularly investigated for spontaneously developing colonies using phase-contrast microscopy. When such colonies were observed, they were passaged to a new well using Trypsin-EDTA (Gibco, Bethesda, MD, USA). Only those growing colonies of cells, which were able to secrete human serum albumin, were further passaged and by this way we have established cell line C, in addition to a number of other cell lines. After trypsinization the immortalized human hepatocytes were resuspended in medium containing 10 % DMSO and 20 % fetal calf serum and stored in liquid nitrogen. Each cryovial contains 5 x 10E6 cells.

TABLE 6

	Cell line C	**Primary human hepatocytes**
Cytokeratin expression (% of cells)		
• CK7	-	-
• CK8	50	60
• CKI8	100	95
Bilirubin conjugation (μmol/μg DNA/24 h)		
• Total	0.47	0.79
• Mono-conjugate	0.23	0.55
• Di-conjugate	0.24	0.24
• Ratio di-/mono	1.04	0.44

Protein secretion (µg/µg DNA/24 h)		
• Human serum albumin	70	120
• Fibrinogen (minus IL-6)	4.1	310
• Fibrinogen (plus IL-6)	41	not done

Table 6 : Cytokeratin expression, protein secretion and bilirubin conjugation of the immortalized human hepatocyte line C in comparison with freshly isolated human hepatocytes.

As shown in table 6 the pattern of cytokeratin (CK) expression of cell line C reveals a low level of CK7 and a high level of CK8 and CKI8 expression, which are characteristic for adult human hepatocytes. Cell line C secretes human serum albumin and the base-line fibrinogen production can be enhanced by interleukin-6 (IL-6) treatment. In addition, the ability to conjugate bilirubin is comparable with that of primary human hepatocytes. Finally, this C cell line was not found to be immunoreactive for the monoclonal antibody HCV.OT 1F.

2.2 Cell culture system

2.2.1 Culture of immortalized human hepatocytes (C-cell line) starting from cryopreserved cells

The following protocol is designed for the culture of immortalized hepatocytes (cell line C) starting from one cryovial containing 5 million hepatocytes.

2.2.1.1 Thaw one cryovial on ice;

2.2.1.2 Resuspend the cells in ice cold 10 ml of William's E (WE)medium (Gibco, Bethesda, MD, USA) at 4°C in a conical tube (Sarstedt, Numbrecht, Germany);

2.2.1.3 Centrifuge for 5 min, at 800 rpm (143 xg) and 4°C in a Mistral 3000i centrifuge (MSE, Leicester, United Kingdom);

2.2.1.4 Discard the supernatant;

2.2.1.5 Resuspend the cells carefully in 10 ml of WE-medium;

2.2.1.6 Centrifuge again for 5 min, at 800 rpm (143 g) and 4°C in a Mistral 3000i centrifuge (MSE, Leicester, United Kingdom);

2.2.1.7 Discard the supernatant;

2.2.1.8 Resuspend the cells carefully in 1 ml of WE-medium supplemented with 10 % Fetal Calf Serum (Gibco, Bethesda, MD, USA), 2 mM L- glutamine (Gibco), 20 mU/ml insulin (Novo Nordisk Pharma), 50 nM dexamethason, 50 µg/ml gentamycin (Schering-Plough), 100 µg/ml vancomycin (Lilly), 2.5 µg/ml fungizone (Bristol-Meyers Squibb) and 100 U/ml penicillin (Continental Pharma) (complete WE-medium);

2.2.1.9 Transfer the cell suspension to a 25 cm2 culture flask (Nunc, Life Technologies, Roskilde, Denmark) and 5 ml complete WE medium is added.

2.2.1.1 0 Culture cells at 37 °C and 5% C02;

2.2.1.11 Change medium every 3 days;

2.2.1.12 Evaluate cell confluency by phase contrast microscopy on a day to day basis;

2.2.1.13 When 90 to 100 % cell confluency has been reached, cells should be trypsinised.

2.2.2 Trypsinisation of monolayer cell cultures (C-cell line)

2.2.2.1 Discard medium from culture flasks;

2.2.2.2 Wash cells 1 time with Phosphate Buffered Saline (PBS);

2.2.2.3 Add 5 ml Trypsin-EDTA (Gibco, Bethesda, MD, USA) to a 25 cm 2 flask or 10 ml to a 75 cm2 flask;

2.2.2.4 Incubate at 37 °C, 5 % C02 for 5 minutes;

2.2.2.5 Detach the cells from the flask by shaking;

2.2.2.6 Add two volumes of cold WE-medium (4 °C) to stop the trypsin activity;

2.2.2.7 Centrifuge the cells for 5 min at 800 rpm and 4°C in a Mistral 3000i centrifuge (MSE, Leicester, United Kingdom);

2.2.2.8. Discard supernatant;

2.2.2.9. Resuspend the cell pellet in complete WE-medium.

2.2.3 Culture of immortalized human hepatocytes (C-cell line) in a Rotating Cell Culture System

The immortalized human hepatocytes are cultured in a Rotating Cell Culture System (RCCS, Synthecon, Houston, USA), which is a vertically rotated, culture vessel with a membrane that allows gas exchange. The cells establish a uniform, low shear, fluid suspension orbit within the vertically rotating culture vessel. In this microgravity system, the absence of damaging stress forces allows three dimensional aggregation of cells (Schwarz RP et al., 1992).

A 50 ml volume RCCS vessel has a 1/2 inch fill port and two smaller syringe ports. Cells are cultured in the RCCS according to the manufacturer's instructions :

2.2.3.1 Transfer the RCCS vessel to a Laminar Flow Hood. Remove the end caps and place them on sterile petri dishes;

2.2.3.2 Wash the vessel with PBS;

2.2.3.3 After trypsinisation (2.2.2.9) dilute the hepatocyte suspension (C-cell line) in complete WE-medium to yield a final concentration of 0.5 x 10E6 cells per ml;

2.2.3.4 Load the cell suspension into the RCCS-vessel through the 1/2 inch port;

2.2.3.5 An empty 5 ml sterile syringe is attached to one of the syringe ports. The syringe valve is opened and gently tap on sides to expel air bubbles from under the port. Close the syringe port.

2.2.3.6 Attach the vessel to the rotator base in a $C0_2$ (5%)-incubator, at 37°C;

2.2.3.7 Turn on power and adjust initial rotation speed of 15 to 20 rpm;

2.2.3.8 As cell aggregates grow, the rotation speed is adjusted to compensate for increased sedimentation rates.

2.2.4 Change medium and harvesting of cell aggregates

2.2.4.1 Turn off power and remove the vessel from the rotator base and take it to a sterile hood;

2.2.4.2 Open the 1/2 inch fill port and remove cell suspension by aspiration with a 25 ml pipet;

2.2.4.3 Centrifuge the cell suspension for 5 minutes at 800 rpm and 4 °C in a Mistral 3000i centrifuge (MSE, Leicester, United Kingdom);

2.2.4.4 Discard supernatant;

2.2.4.5 Take cell aggregates with a sterile glass pipet from the cell pellet. For immunocytochemistry (see 2.5) immerse the cell aggregates in liquid nitrogen chilled 2-Methylbutane (Sigma-Aldrich, Steinheim, Germany) and store at -20 °C;

2.2.4.6 Resuspend the remaining cell pellet in complete WE-medium. Load the cell Suspension into the RCCS-vessel and proceed according to the protocol discussed above (2.2.3.5 - 2.2.3.8).

2.3 Source and preparation of the inoculum

The inoculum was derived form the liver of patient CD, who underwent an orthotopic liver transplantation (OLT) for end stage chronic liver disease, most probably due to chronic infection with non-A-G-agent. Patient CD is a man of 39 years old who presented with recurrent oesophageal variceal bleeding as a complication of portal hypertension due to cirrhosis in december 1995. The cirrhosis could not be attributed to any known viral, toxic or metabolic cause, except for a positive immunoreactivity to HCV.OT 1F on a liver biopsy. In march 1996 he underwent an orthotopic livertransplantation. The explanted liver showed a micronodular type of cirrhosis, with pronounced mononuclear cell infiltrates in the portal tracts and the immunoreactivity to monoclonal antibody HCV.OT 1F was confirmed. Since all serological markers of known viral infections were repeatedly negative, including serum anti-HCV and HCV-RNA, this patient was considered of having HY.

2.3.1 Cryopreserved non-A-G infected primary human hepatocytes

The left lobe from the explanted liver was used for isolation of the hepatocytes by a method as described above, which were subsequently cryopreserved (inoculum A).

2.3.2 Homogenate of cryopreserved NON-A-G infected primary human hepatocytes

Ten million cryopreserved human hepatocytes were homogenized with a Dounce homogenizer, which yielded inoculum B. Inoculum B was separated in a supernatant (inoculum C) and pelleted fraction (inoculum D) by centrifugation (250 x g for 10 minutes, 4°C).

2.3.3 Homogenate of NON-A-G infected ilver tissue

Liver tissue from this patient, which was deep frozen in liquid nitrogen immediately after OLT, was homogenized and fractionated, according to the following protocol:

2.3.3.1 Thaw 2.0 gram of frozen liver tissue on ice in 20 ml ice-cold buffer TENB (0.05 M Tris, 0.001 M EDTA, 0.1 M NaCl; pH 7.5);

2.3.3.2 Homogenize sequentially by the Polytron (5 x 1 minute) and 4 times by a Dounce homogenizer;

2.3.3.3 Centrifuge the homogenate at 250 gfor 10 mm (4°C) and discard the pellet;

2.3.3.4 Centrifuge the supernatant from the previous step at 12,000 x *g* for 20 min (4°C) and discard the pellet;

2.3.3.5 Dilute the supernatant 6 x in TENB

2.3.3.6 Centrifuge the solution at 251,800 x g for 2 hours (20°C).

2.3.3.7 Dilute the supernatant from step 2.3.3.6 10 x in complete WE medium (inoculum E);

2.3.3.8 Resuspend the pellet from step 2.3.3.6 in 15 ml of TENB. Inoculum F consisted of 5 ml of this solution.

2.4 Incubation of the immortalized human hepatocytes with inoculum in the RCCS

The immortalized human hepatocytes were cultured in 6 RCCS vessels (25 x 10E6 cells/vessel) and incubated with the different NON-A-G inocula (A-F):

2.4.1 Inoculum A : 10 x 10E6 cryopreserved NON-A-G infected primary human hepatocytes (see 2.3.1);

2.4.2 Inoculum B : total homogenate of 10 x 10E6 cryopreserved NON-A-G infected primary human hepatocytes (see 2.3.2);

2.4.3 Inoculum C : supernatant fraction of inoculum B (after centrifugation for 10 minutes, at 250 x g) (see 2.3.2);

2.4.4 Inoculum D : pelleted fraction of inoculum B (after centrifugation for 10 minutes, at 250 x g) (see 2.3.2);

2.4.5 Inoculum E : supernatant fraction of homogenized NON-A-G infected liver tissue (see 2.3.3.7);

2.4.6 Inoculum F : pelleted fraction of homogenized NON-A-G infected liver tissue (see 2.3.3.8).

Medium was changed and cell aggregates were harvested every 3 to 4 days, starting at day 2 after infection.

2.5 Immunocytochemistry

Immunocytochemistry was performed as described previously (Yap SH et al., 1994) using a three-step indirect immuno-peroxidase procedure:

2.5.1 Cut sections (4 μm thick) of fresh frozen materials and place on slides as for routine histological examination

2.5.2 Fix in acetone for 10 minutes at room temperature. Then air dry;

2.5.3 Rinse with distilled water and place in phosphate buffered saline (PBS) pH 7.2 for 5 minutes

2.5.4 Incubate for 30 minutes with the mouse monoclonal antibody HCV.OT 1F (purified IgG_1 : 50 ng /μl). The monoclonal antibody was pre-incu bated overnight with the homogenate of normal human liver tissue before use

2.5.5 Tap off antibody and place slide in PBS bath for 5 minutes;

2.5.6 Incubate for 30 minutes with peroxidase conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:50 in PBS;

2.5.7 Tap off antibody and place slide in PBS bath for 5 minutes;

2.5.8 Incubate for 30 minutes with peroxidase conjugated swine anti-rabbit immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:100 in PBS;

2.5.9 Tap off antibody and place slide in PBS bath for 5 minutes;

2.5.10 Incubate for 10 minutes in 100 mM acetate buffer (pH 5.2), containing 0.05 % 3-amino-9-ethyl-carbazole and 0.01 % H_2O_2;

2.5.11 Rinse with distilled water;

2.5.12 Counterstain and mount with coverslip.

2.5.13 Evaluate microscopically for bright red granular staining of immunoreactive sites in the cytoplasm of the hepatocytes.

3. Results

3.1 Co-culture of the C-cell line with NON-A-G infected cryopreserved primary hepatocytes (inoculum A)

Two days after initiation of the co-culture some immuno-reactivity to HCV.OT 1F was seen in a minority of viable cells. However, eight days later this HCV.OT 1F

immunoreactivity was even more pronounced. The latter finding suggested that the immortalized human hepatocytes acquired HCV.OT 1F immunoreactivity when they were co-cultured with HCV.OT 1F immunoreactive primary hepatocytes. However, in order to exclude that the HCV.OT 1F immunoreactive cells were surviving primary hepatocytes and not immortalized hepatocytes, additional experiments with incubation of homogenized tissue or cells were performed.

3.2 Incubation of the C-cell line with total (inoculum B) and fractionated (inoculum C and D) homogenate of NON-A-G infected cryopreserved primary hepatocytes

Four days after start of the incubation of the C-cell line with the total homogenate (inoculum B) a typical granular immunoreactivity for HCV.OT 1F was seen in a minority of cells, which increased at day 8 and 12. The staining remained positive until day 24, after which the HCV.OT 1F immunoreactivity was lost.

At day 8 after infection with the supernatant fraction (inoculum C) of the cell homogenate a transient granular positivity was observed in the harvested hepatocytes, which was lost at the subsequent time points. However, at day 29 and 33 some positivity was observed again in a minority of cells. After infection with the pelleted fraction (inoculum D) of the cell homogenate no positivity for HCV.OT 1F could be seen until day 47 after infection, which adds to the specificity of the observed staining in the parallel experiments.

3.3 Incubation of the C-cell line with fractionated homogenate of NON-A-G infected liver tissue (inoculum F and F)

Twelve days after incubation with the supernatant fraction of the liver homogenate (inoculum F) a marked HCV.OT 1F immunoreactivity was observed, which was maintained at least until day 24 (last sample evaluated).

After incubation with the pelleted fraction (inoculum F) it took 21 days for some cells to gain HCV.OT 1F immunoreactivity, which was less pronounced than the cells in the parallel experiment. It should be noted that the viability of the cells was less after incubation with inoculum F, which may have been too concentrated or toxic for the cells.

4. Conclusions

The findings of this bio-assay demonstrate that anti-HCV negative, serum HCV-RNA negative, HCV.OT 1F immunoreactive chronic hepatitis is caused by a transmissible agent. The successful infection of immortalized human hepatocytes by homogenate of NON-A-G infected liver tissue or hepatocytes in the rotatory cell culture system, represents an ideal test system (bio-assay) to characterize and isolate the infectious agent.

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EXAMPLE 6

ISOLATION AND CHARACTERIZATION OF POLYNUCLEOTIDE SEQUENCES OF THE ETIOLOGICAL AGENT OF HEPATITIS Y

Previous experiments revealed that a transmissible agent might be contained in sera or plasma or liver of patients with non-A-G hepatitis (see above). As a working hypothesis we assumed that such agent might be a virus and provisionally termed it Hepatitis Y virus (HYV). In order to isolate nucleic acid from HYV the following experiment was conducted.

Representational Difference Analysis (RDA)

Overview of the method

The protocol begins with the isolation of the total nucleic acid of the tester samples (presumed HepY positive material) and the driver samples (Hep Y negative material). The isolation was performed using the Boom method. The total nucleic acid content was converted into cDNA using random hexamers as non-specific primers. The resulting cDNA was cut by the restriction enzyme Sau3A I, which has a recognition site of four nucleotides (GATC). Using these Sau3A I sites adapters were ligated onto the cDNA molecules. PCR reactions were performed with adapter primers resulting in 'representations', of both tester- and driver-material. The tester representation was restricted by Sau3A I to remove the adapters and new adapters with a different sequence were ligated onto this representation. Tester and driver material were mixed, a hybridisation reaction was performed, followed by a PCR reaction with the same adapter primers as ligated to the tester representation. Therefore only tester-tester hybrids were presumed to be amplified exponentially by these primers. The material obtained after the first subtractive hybridisation was called Difference Product 1 (DP1). Adapters were removed from the amplified testermaterial by Sau3A I digestion and different

adapters were ligated to the testermaterial only. By changing the adapter primers of the testermaterial a couple of times, it was possible to perform a number of subtractive hybridisation's, thereby enriching tester specific sequences. Finally, the remaining tester material was isolated and purified from gel, cloned into a pGem4Z-vector by using the Sau3A I sites, and sequenced. These sequences were compared with different sequence databases. Sequences that remain unknown were used to design PCR and/or NASBA primers. These sequences were tested on negative and positive control genomic DNA samples and those sequences with no genomic DNA homologues were further investigated.

Example 1: Detailed protocol

1) Tester preparation

Tester samples: - patient OHN serum December 1996
- patient OHN serum August 1997
- Macaque 1FU serum 21/1/97
- Chimp Sylvia plasma 4/2/97

(The patient and the animals, from which these samples were obtained, were at least once positive by immunostaining of liver sections with the HCV.OT 1F monoclonal antibody)

Total nucleic acid from 1 ml serum was isolated using the "Boom" method (Boom R et al, 1990; J Clin Microbiol.28: 495-503) with 9 ml of lysisbuffer (pH 6.2, 4.7 M Guanidine isothiocyanate, Triton X-100, Tris/HCl)

After elution in 100 µl DEP-C-treated water(Rnase, Dnase free, Ambion) the nucleic acid was precipitated overnight at a temperature of -20 °C, by adding 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample was centrifuged during one hour at 25,000 rpm at 4 °C (120,000xg; Beckman Sw 28 rotor).

After air-drying, the pellet was dissolved in a mixture of 0.2 µl RNase inhibitor (40 units/ul) and 4.8 µl DEP-C-treated water.

2) Driver preparation

Driver sample: - Livertissue L701

(This sample was obtained from a human liver not reacting with the monoclonal antibody HCV.OT 1F)

A few slices of liver (about 10 mg) were isolated using the "Boom" method with 9 ml of lysisbuffer (4.5.M Guanidine isothiocyanate, pH 6.2).

The total nucleic acid content was eluted in 100 µl DEPC-treated water.

3) First strand synthesis

To the 5 µl tester or driver sample 0.7 µl of DEPC-treated water and 0.3 µl of random hexamers (50 ng/µl) were added.

This mixture was incubated during 10 minutes at 70 °C and next during 1 minute at 4 °C.

The following was added:
- 1 µl of 10 X RTbuffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl)
- 1 µl of a 25 mM $MgCl_2$ solution
- 0.5 µl of a 10 mM dNTP solution
- 1 µl of 0.1 M DTT.

This mixture was incubated during 5 minutes at a temperature of 25 °C and then 0.5 µl of Superscript II enzyme (200 units/µl, RNase H negative reverse transcriptase , Gibco BRL) was added.

Incubation conditions:

- 10 minutes at 25 °C.
- 50 minutes at 42 °C.
- 15 minutes at 70 °C.

4) Second-strand synthesis

Immediately after the first strand reaction the following reagents were added to the reaction mixture:

- 8 µl of 2.5 X second strand buffer (100 mM Tris-HCl (pH 7.2), 225 mM KCl, 7.5 mM $MgCl_2$, 7.5 mM DTT)

- 2 µl of DNA polymerase I (13.6 units)

- 0.5 µl of RNase H (1 unit)

Incubation conditions:

- 120 minutes at 14 °C.

- 10 minutes at 70 °C.

and then 0.5 µl of T4 DNA polymerase (4 units) was added.

This mixture was incubated at 37 °C. for 10 minutes.

The reaction was purified by a phenol/chloroform extraction.

An overnight precipitation (-20 °C) was performed after adding 0.1 volume of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample was centrifuged for 1 hour at 25000 rpm at 4 °C.

After drying, the pellet was dissolved in 16 µl of DEPC-treated water.

5) Sau3A I digestion

Digestion with the enzyme Sau3A I was performed in a total volume of 20 µl:

- 16 µl of ds cDNA

- 2 µl of 10 X One Phor All buffer (100 mM Tris acetate (pH 7.5), 100 mM MgAcetate, 500 mM KAcetate)

- 2 µl of Sau3A I (8 units/µl)

This reaction was incubated overnight at a temperature of 37 °C.

The reaction was purified by a phenol/chloroform extraction and an overnight precipitation (-20 °C) was performed by adding 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample was centrifuged for 1 hour at 25000 rpm at 4 °C.

After drying, the pellet was dissolved in 1 µl of DEPC-treated water.

6) Adapter ligation

For the adapter ligation the Rapid Ligation kit (Boehringer Mannheim) was used.

To the 1 µl sample the following reagents were added:

- 0.8 µl adapter mix (containing 0.5 µg of R-Bgl-12 and 0.25 µg of R-Bgl-24)
- 0.5 µl of DNA dilution buffer (5X)
- 2.5 µl of T4 DNA ligase buffer (2X)
- 0.3 µl of T4 DNA ligase

Incubation conditions:

15 minutes at room temperature

1 hour at 16 °C.

The sequences of the adapters were:

R-Bgl-12 5'- GATCTGCGGTGA-3'

R-Bgl-24 5'- AGCACTCTCCAGCCTCTCACCGCA-3'

The following product is formed:

```
5'-AGCACTCTCCAGCCTCTCACCGCAGATCXXXXXXXXX-R-Bgl-12 -3'
                    3'- AGTGGCGTCTAGYYYYYYYYYCTAG-R-Bgl-24 -5'
```

where XXXXX resp. YYYYYY are the target sequence and its complement.

7) Generation of the representations

For each sample, four 100 µl PCR reactions were performed.

The products of the ligation reactions were diluted ten times in DEPC-treated water (input material).

For each PCR reaction the following reagents were mixed:

- 79.5 µl of DEPC-treated water
- 10 µl of 10X PCR buffer (Perkin Elmer, 100 mM Tris-HCl, pH8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% (w/v) gelatine)
- 8 µl of a 2.5 mM dNTP-solution
- 1 µl of R-Bgl-24 adaptor-primer (1 µg/µl)
- 0.5 µl of input material

The sequence of the adaptor-primer was:

5'- AGCACTCTCCAGCCTCTCACCGCA-3'

This mixture was incubated for 3 minutes at a temperature of 72 °C and then to each reaction 1 µl of Amplitaq (5U/µl , Amplitaq DNA Polymerase, recombinant, thermostable, 94 kDa DNA Polymerase, Perkin Elmer) was added.

Incubation conditions:

- 5 minutes at 72 °C.
- 30 cycles: 1 minute; 95 °C.
  3 minutes; 72 °C.
- 10 minutes at 72 °C.

After these PCR reactions the representations were analysed on a 1.25% agarose gel. DNA yield was estimated by using the extinction at 260 nm(OD260).

8) Removing of adapter sequences

Both tester and driver representations were digested with Sau3A I to remove the adapters.

Ten µg of the tester representations was digested with 16 units Sau3A I in a total volume of 40 µl (see par.5) and hundred µg of the driver representation was digested with 64 units Sau3A I in a total volume of 200 µl.

The digestion's were performed overnight at a temperature of 37 °C.

The digested driver representation was purified by a phenol/chloroform extraction, followed by an overnight precipitation at -20 °C., after adding 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample was centrifuged for 15 minutes at 13000 rpm () at 4 °C. After drying the pellet was dissolved in 50 µl of DEPC-treated water, DNA yield was calculated to be 1.058 µg/µl by using the OD260 value.

9) Purification of the tester representation

Of the tester representations 200-600 bp fragments were isolated from an 1.5% agarose gel and purified by silica binding in the presence of high salt using the Qiagen Qiaex II Agarose Gel Extraction kit.

The samples were eluted in 40 µl of DEP-C-treated water.

The Qiaex eluates were precipitated by adding 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol, followed by an overnight incubation at a temperature of -20 °C.

The samples were centrifuged for 15 minutes at a speed of 13,000 rpm (Heraeus Sepatech table centrifuge) at 4 °C.

After drying the pellets, they were dissolved in 1 µl of DEP-C-treated water.

10) Ligation of new adapters to tester material

For the adaptor ligation the rapid ligation kit of Boehringer Mannheim was used (see also par.6).

The following reagents were mixed:

- 1 µl of 'Qiaex cleaned' tester DNA (see par.9)
- 2.4 µl of 0.8 µl J adapter mix, containing 0.5 µg of J-Bgl-12 and 0.25 µg of J-Bgl24
- 1 µl of 5X DNA dilution buffer
- 5 µl of 2X DNA ligase buffer
- 0.6 µl of T4 DNA ligase

Incubation conditions:

15 minutes at room temperature

1 hour at 16 °C.

The sequences of the adapters were:

J-Bgl-12 5'-GATCTGTTCATG-3'

J-Bgl-24 5'-ACCGACGTCGACTATCCATGAACA-3'

After ligation the samples were diluted to a concentration of about 10 ng/µl.

11) Subtractive hybridisation

As driver material in the hybridisation reaction the following mixture was used:

- 8.5 µg of cDNA from liver L701, isolated in step 8.
- 11.5 µg of Human Cot-1 DNA (Gibco BRL)

As tester material 400 ng of adaptor ligated tester material was used, leading to a proportion of driver and tester in this hybridisation of 50:1 (w/w).

The driver and tester material was combined and 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol were added.

Precipitation: - 1 hour at - 70 °C.

- 5 minutes at 37 °C.

The samples were centrifuged for 15 minutes at 13000 rpm() and 4 °C.

After drying the pellets, they were dissolved in 4 µl 3XEE buffer (30 mM EPPS, pH 8.0; 3 mM EDTA)

Incubation conditions:

: - 5 minutes at 37 °C.

- 5 minutes at 98 °C.

After reducing the temperature to 67 °C., 1 µl of 5M NaCl was added.

This mixture was incubated for 20 hours at a temperature of 67 °C. Afterwards 395 µl of DEPC-treated water was added and the sample was stored at a temperature of -70 °C.

12) Generation of first difference products (DP1)

For each sample, four 100 µl PCR-reactions were performed as described below:

- 60 µl of DEPC-treated water
- 10 µl of 10X PCR buffer (Perkin Elmer, 100 mM Tris-HCl, pH8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% (w/v) gelatine)
- 8 µl of 2.5 mM dNTP-solution
- 20 µl of subtractive hybridisation product

This mixture was incubated for 3 minutes at 72 °C, 1 µl of Perkin Elmer Amplitaq (5 U/µl) was added, followed by an incubation for 5 minutes at 72 °C.

Then 1 µl J-Bgl-24 adaptor primer (1 µg/µl) was added.

Incubation conditions:

- 10 cycles 1 minute 94 °C.
  3 minutes 70 °C.
- 10 minutes 72 °C.

After pooling the four reactions, the samples were purified by performing a phenol/chloroform extraction.

The samples were precipitated for 1 hour at a temperature of -70 °C., after adding 0.1 volumes of 3M NaOAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The samples were centrifuged for 15 minutes at 13000 rpm at 4 °C.

After drying the pellets they were dissolved in 20 µl of DEPC-treated water.

To remove ssDNA molecules a treatment with mung bean nuclease was performed.

To each reaction the following reagents were added:

- 15 µl of DEPC-treated water
- 4 µl of 10X mung bean nuclease buffer 100 mM NaOAc, (pH 5.0), 1mM ZnOAc, 10 mM L-cysteine, 500 mM NaCl, 50 % glycerol.
- 1 µl of mung bean nuclease (34 units/µl)

This mixture was incubated for 35 minutes at a temperature of 30 °C and then 160 µl of 50 mM Tris-HCl pH 8.9 was added.

This was incubated for 5 minutes at 98 °C.

For the secondary PCR-reactions (four reactions for each sample) the following reagents were mixed:

- 60 µl of DEPC-treated water
- 10 µl of 10X PCR buffer (Perkin Elmer, 100 mM Tris-HCl, pH8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% (w/v) gelatine)
- 8 µl of 2.5 mM dNTP solution
- 1 µl J-Bgl-adaptor-primer (1 µg/µl)

To this 20 µl of the mung bean nuclease treated product was added and the mixture was incubated for 1 minute at 95 °C.

After cooling the mix to 80 °C. 1 µl of Amplitaq (PE, 5 units/µl)) was added.

Incubation conditions of the following PCR:

- 20 cycles 95 °C., 1 minute
  70 °C., 3 minutes
- final cycle 72 °C., 10 minutes

After the PCR the reactions were pooled and 10 µl was analysed on a 1.5% agarose gel; the remainder was purified by a phenol/chloroform extraction.

After addition of 0.1 volume of 3M NaOAc (pH5.3) and 2.5 volumes of ice-cold 96% ethanol, the samples were precipitated overnight at -20 °C.

After centrifugation at 13000 rpm ()at room temperature the pellets were airdried and dissolved in 50 µl DEPC-treated water.

DNA yield was calculated using the OD260 values.

13) Generation of DP2

For the generation of DP2 the same protocol (steps 8 -12) was used as described for the generation of DP1. After replacing the tester adapter with the N-Bgl adaptor, a new subtractive hybridisation was performed.

The N-adaptor sequences are:

N-Bgl-12 5'-GATCTTCCCTCG-3'

N-Bgl-24 5'-AGGCAACTGTGCTATCCGAGGGAA-3'

The proportion driver:tester was 100:1.

As tester material 250 ng of the ligated DP1-material was used and as driver material a mixture of 6.5 µg cDNA from liver L701 and 18.5 µg of human Cot-1 DNA. For the PCR with the N-Bgl-24 primer an annealing temperature of 72°C was used.

14) Generation of DP3 (SB98018)

For the generation of DP3 the same principle was used as described for the generation of DP1. After replacing the N-Bgl tester adapter with J-Bgl adaptor, a new subtractive hybridisation was performed.

For the generation of DP3 the driver:tester proportion was 800:1

As testermaterial 3.1 ng of the ligated DP2-material and as drivermaterial a mixture of 7 µg liver cDNA from liver L701and 18 µg of human Cot-1 DNA was used. For the PCR with the J-Bgl-24 primer an annealing temperature of 70°C was used.

15) Analysis of the DP3-products

Bands of interest in the DP3 products were isolated from a 1.5% agarose gel (in 1xTAE buffer) and purified using the Qiaex II gel extraction kit (Qiagen).

A volume of 0.5 ul of these extracts was inserted in the pGEM T-vector (Promega). After ligation, the ligation products are 10x diluted in water and electroporated in MC1061 electrocompetent E.Coli cells. Products are plated on LB agar plates with Ampicillin (500:1). Plates are incubated overnight at 37°C. The insert colonies were screened on insert length with SP6 and T7 primers. The plasmids containing inserts were sequenced with SP6 and T7 promoter sequences as primers.

The inserts between two consecutive Sau3A I sites were investigated separately. The sequences presumed to be derived from genomic sequences not related to hepatitis Y were discarded.The following criteria were used:

- >90% homology of the insert to known sequences **or**

- positive reaction in PCR using primers derived from the insert sequence on human genomic DNA yielding fragments having a length comparable to the PCR product of the plasmid containing the insert.

Of the hundred sequences analysed two sequences, both derived from OHN serum did not fulfil one of these criteria and were further analysed.

- sequence nd1.1b (derived from OHN serum December 1996)

- sequence nd2.1 (derived from OHN serum August 1997)

EXAMPLE 7

Utility of RT-PCR using nested primer set and probe derived from SEQ ID-1

PROTOCOL RT-PCR

RNA extraction.

RNA was extracted from 200 µl serum or EDTA treated plasma, in a single step acid guanidinium thiocyanate-phenol-chloroform extraction procedure, as described by Chomczynski & Sacchi (*Anal Biochem* 1987; 162:1 56-159). After isopropanol precipitation, the RNA was dissolved in 8µl DEPC-treated water.

HYV detection by RT-PCR assay.

For reverse transcription, the RNA was denatured by incubation for 10 min at 60° C. To the RNA fraction, 12µl reverse-transcription mix was added, containing 200 units Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Gibco BRL, Bethesda, MD), 30 units placental RNase inhibitor (Pharmacia, Uppsala, Sweden), 80 pmol random primers (Boehringer Mannheim, Germany), 0.5 mM of each deoxynucleotide triphosphate (dNTP; Pharmacia), 10 mM dithiotreitol, an appropriate volume of 5 x reverse transcriptase buffer, and DEPC treated water. The cDNA synthesis was carried out at 37°C for 1 hour and stopped by heating in boiling water for 1 minute. Subsequently, double round nested PCR was performed. Briefly, the first PCR mixture contained 5 µl cDNA solution, 16 pmol of each HYV (first round) primer, 0.2 mM of each dNTP and 1 unit Primezyme DNA polymerase (Biometra, Gottingen, Germany) in a total volume of 50 µl PCR buffer (10 mM Tris-HCl pH

8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin). Samples were overlaid with mineral oil and subjected to thermal cycling. For the nested PCR, 5 µl of first round PCR product was added to a new PCR mixture containing the appropriate nested PCR primers for a second amplification. First and second round cycling conditions were 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, 35 cycles. Amplified DNA was visualized by agarose gel electrophoresis (2%) and ethidium bromide staining.

RESULTS

We analysed the inocula used in the bioassay studies. Supernatant fraction S3 which resulted in a positive HCV.OT 1F staining in the immortalized hepatocytes was tested for the presence of sequenceSEQ ID-1 by nested PCR. In a parallel experiment, a pellet of liver homogenate (P2) was also investigated, which was shown to give negative results in the bioassay studies.

- Supernatant fraction S3: +/+
- Pellet fraction P2 -

2. Detection of sequenceSEQ ID-1 in the patient population:

-hepatitis non A-G infected patients: 7/10 were found positive for sequence SEQ ID-1

YI : OHN plasma 7/97	+/+/-/-
OHN serum *3/97*	+
OHN serum *12/96*	-
Y2: CD *12/95*	+/+
Y3: VG *8/97*	+
Y4: RG *9/98*	*+1-*
Y5: CJ *3/96*	+
Y6 : AL *10/95*	-
Y7: ML *4/95*	-
Y8: BW	-/-

Y9 : CM *3/98* +

Y10 MG *1/96* +

negative control patients (HCV.OT 1F negative): 0/7 positive for sequence SEQ ID-1

NI : DGu (toxic hepatitis) *8/97* -/-

N2: DGr (toxic hepatitis) *4/96* -

N3: LRe (toxic hepatitis) *5/95* -

N4: HCV.OT 1F negative donorliver -/-

N5 :VP (HBV infected) -

N6: HH (HBV infected) -

N7: LRi (HBV infected) *4/98* -

3. Detection of sequence SEQ ID-1 in healthy blood donors:

- 2 of 28 blooddonors tested positive for sequence SEQ ID-1
- BD 44(274-6) en BD 53 (274-15): +

EXAMPLE 8

Cesium chloride gradient ultracentrifugation fractions monitored by nested RT-PCR for HYV related sequence SEQ ID-1

Materials and methods

- Buffer G:

10 mM Hepes pH 7.4

75 mM KCl

5 mM $MgCl_2$

500 mM NaCl

- Gradient solutions: 26 % $CsCl_2$ and 58 % $CsCl_2$ in buffer G
- Autoclave cesium chloride solutions
- Preparation of gradient in autoclaved RNase free centrifugation tubes

- Gradient 1: control TENB

Gradient 2: idem gradient 1

Gradient 3: 0.5 ml supernatant S3 of liver homogenate (of patient CD,see example 5)

Gradient 4: idem gradient 3

Gradient 5: 0.5 ml OHN plasma (7/97)of HY patient OHN

Gradient 6: idem gradient 5

- Centrifugation: Sw50 rotor(Beckman) , 40,000 rpm, 74 hours, 20°C, 250,000x g.
- Fractions were taken from each tube starting from bottom to top
- Dilution of the sample 5x by proteinase K solution (1mg/ml)
- isopropanol extraction
- cDNA synthesis (starting from all extracted material)
- First round PCR using all cDNA (primers SEQ ID-NO 3, SEQ ID NO: 4)
- Second round PCR using 10 µl PCR product from the first round (primers: SEQ ID NO: 5 and SEQ ID NO: 6.)

RESULTS

The results are shown in figs 5 and 6. In conclusion it can be stated that the PCR positive fractions are found around a density of 1.22 g/ml. Positive fractions at the top or bottom of a gradient most likely are due to different artifacts (particles floating in lipid-rich complexes resp. naked nucleic acid from degraded particles which is having a higher density).

EXAMPLE 9

Isolation of the complete HYV genome

In order to isolate the complete viral genome the following two methods can be employed: - Rapid amplification of cDNA ends (RACE)

-'Modified RDA (Representational difference Analysis)'

1 Rapid amplification of cDNA ends (RACE)

Overview of the protocol

For this approach the Clontech Marathon TM cDNA Amplification Kit is used. This is a method for performing both 5' and 3' rapid amplification of cDNA ends from the same template.

The viral RNA is converted into ds cDNA , primed by a random hexamer and the ds cDNA is blunt-ended ligated to the Marathon cDNA Adaptor. Next, this uncloned library of adaptor-ligated ds cDNA is used as input material to perform 5'- and 3' RACE PCR reactions. These PCR reactions are performed with an internal gene specific primer (GSP) and the adaptor primer (AP1).

The RACE products may be characterised on an ethidiumbromide-stained agarose gel. Bands of interest are isolated from gel, purified and cloned into a suitable vector by blunt-end ligation.Alternatively one of the specific cutting sites in the adaptorsequence may be used.

As input material for the RACE reactions the following cesium-chloride gradient fractions,as descibed in example 8, may be used:

Gradient IV (S3-supernatants) - fraction G-IV-7 (density 1.311g/ml)
- fraction G-IV-11 (density 1.226 g/ml)

Gradient V (OHN plasma 7/97) - fraction G-V-1 (density 1.359g/ml)
- fraction G-V-2 (density 1.356 g/ml)
- fraction G-V-4 (density 1.334 g/ml)
- fraction G-V-5 (density 1.337 g/ml)
- fraction G-V-11 (density 1.225 g/ml)
- fraction G-V-12 (density 1.207 g/ml)

Detailed protocol

* Sample preparation

The samples are isopropanol precipitated at a temperature of -20 °C.

Samples are spinned down in a table centrifuge during 30 minutes by 14000 rpm and a temperature of 4 °C.

Pellets are washed with cold 70% ethanol, followed by 5 minutes centrifugation by 14000 rpm and a temperature of 4 °C.

Pellets are dried during 5 minutes in a vacuum-drier and thenredissolved in 25 µl of a solution of DEPC-treated water and a scent of RNA guard (Amersham-Pharmacia Biotech).

When the pellets are redissolved well, they are pooled and a phenol/chloroform extraction is performed.

After the extraction, 1/10 volume of 3M NaAc (pH 5.3), 2 µl of PELLET PAINT TM co-precipitant(coloured glycogen)(Novagen) and 2.5 volumes of 96% ethanol (cold) is added and the sample is precipitated overnight at a temperature of -20 °C.

Samples are spun down in the ultracentrifuge during 1 hour at a speed of 25,000 rpm and a temperature of 4 °C.

After removing the supernatant, the pellet is dried during 10 minutes at roomtemperature and redissolved in 5 µl DEPC-treated water.

* First-strand synthesis

To the 5 µl eluate 0.7 µl of DEPC-treated water and 0.3 µl of random hexamer (50 ng/µl) is added.

Incubation: - 10 minutes at 70 °C

- 1 minute at 4 °C.

Next the following is added: - 1 µl of 10 X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl)

- 1 µl of a 25 mM MgCl2 solution

- 0.5 µl of a 10 mM dNTP solution

- 1 µl of 0.1 M DTT.

This mixture is incubated during 5 minutes at a temperature of 25 °C and then 0.5 µl of Superscript II enzyme (Gibco/BRL) is added.

Incubation: - 10 minutes at 25 °C.

- 50 minutes at 42 °C.

- 15 minutes at 70 °C.

After this incubation the reaction is stored on ice and followed immediately by the second-strand synthesis.

* Second-strand synthesis

To the first-strand synthesis mixture (10 µl in total) the following is added:

- 48.4 µl of sterile, deionized water

- 16 µl of second strand buffer

- 1.5 µl of a 10 mM dNTP-mixture

- 4 µl of second-strand enzyme cocktail (*E. coli* DNA polymerase I (150 U), *E. coli* DNA ligase (30 U) and *E. coli* RNase H (6.25 U))

This is incubated during 90 minutes at a temperature of 16 °C And then2 µl (10 units) of T4 DNA polymerase is added.

Incubation is for 45 minutes at a temperature of 16 °C.

The reaction is purified by a phenol/chloroform extraction.

An overnight precipitation (-20 °C) is performed by adding 0.1 volumes of 3M NaAc (pH 5.3), 0.2 µl (2 µg) of tRNA carrier and 2.5 volumes of ice-cold 96% ethanol.

The sample is spundown in the ultracentrifuge during 1 hour at 25000 rpm and a temperature of 4 °C.

The pellet is air-dried during 10 minutes and redissolved in 5 µl of DEPC-treated water.

* Adaptor ligation

To the 5 µl of cDNA the following is added:

- 2 µl of Marathon TM cDNA adaptor (10 uM)

- 2 µl of 5 X DNA ligase buffer (250 mM Tris-HCl (pH 7.8), 50 mM MgCl2, 5 mM DTT, 5 mM ATP, 25 % w/v Polyethyleneglycol Mw 8,000)

- 1 µl (400 units) of T4 DNA ligase (Promega)

After mixing and spinning down, the ligation-reaction is carried out overnight at a temperature of 16 °C.

Next the reaction is incubated at a temperature of 70 °C for 5 minutes to inactivate the T4 DNA ligase.

The reaction is stored at a temperature of -20 °C.

* Amplification of cDNA Ends (RACE)

For each PCR reaction the following reagents are mixed:

- 36 µl of water

- 5 µl of 10 X cDNA PCR reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl)

- 1 µl of a 10 mM dNTP mix
- 1 µl of Advantage cDNA Polymerase mix (50 X)(Clontech)

For the 5' RACE reaction the following reagents are mixed:

- 43 µl of the above described mixture
- 1 µl of a 10 mM adaptor primer (AP1)
- 1 µl of a 10 mM antisense gene-specific primer (GSP1, p554)
- 5 µl of the adaptor-ligated cDNA.

For the 3' RACE reaction the following reagents are mixed:

- 43 µl of the above described mixture
- 1 µl of a 10 mM adaptor primer (AP1)
- 1 µl of a 10 mM sense gene-specific primer (GSP2, p553)
- 5 µl of the adaptor-ligated cDNA

The following PCR reactions are performed (in a hot-lid thermal cycler):

- 94 °C for 30 seconds
- 5 cycles: 94 °C 30 sec
  72 °C 4 min
- 5 cycles: 94 °C 30 sec
  70 °C 4 min
- 25 cycles: 94 °C 30 sec
  68 °C 4 min

After performing the PCR reactions 5 µl of the PCR-product is analysed on a 1.2% agarose gel. Bands or smears of interest are cut out of gel.

If the primary PCR reaction fails to give enough product to analyse on an agarose gel, a secondary, nested PCR,can be performed using a nested adaptor primer (AP2) and a nested gene-specific primer (NGSP, for 5'RACE: p550, for 3"RACE: p549).

In this case the product of the primary PCR reaction is diluted 50 times in water.

Of this dilution 5 µl can be used as input. The same PCR scheme can be used as described above.

After performing this nested PCR, the samples are again analysed on a 1.2% agarose gel.

Bands or smears of interest are cut out of the gel.

* Analysis of the RACE products

These products are purified by using the Qiagen Qiaex gel extraction kit).

The purified material is analysed on agarose gel.

The DNA is then cloned into a conventional vector by using one of the restriction sites in the marathon adaptor or in a T/A cloning vector.

After cloning, the inserts of the clones are sequencedusing standard methods.

The obtained sequences are screened in sequence databases.

A number of the obtained sequences remain unknown and contain that part of the clone that was used to design the gene-specific primers; these are further analysed in PCR and/or in NASBA.

The sequences are used to design new gene-specific primers.

With these new gene-specific primers, new RACE reactions are performed, the resulting fragments cloned and analysed, until no further extending sequences are found.

2. 'Modified RDA'

Overview of the protocol

The protocol begins with the isolation of the total nucleic acid of a serum sample by using the Boom method. The RNA is converted into DNA by performing a cDNA synthesis reaction using a random hexamer as a non-specific primer. The obtained cDNA is cut by the restriction enzyme, for instance BamH1, used as an example here but other restriction enzyme can be chosen, to generate DNA-fragments with a BamH1-site on both ends. Using these BamH1 sites, partially double-stranded adaptors are ligated onto the cDNA molecules. PCR is performed using the adaptor-primer to amplify this cDNA non specifically. The products are ligated into the T/A vector (Promega) or after restriction with Bam H1 into a BamH1 restricted pGem vector (Promega) and PCR is performed with 2.1 specific primers to detect the presence of the original sequence. With a combination of a gene specific primer, either forward or reversed, and vector specific primers (both SP6 and T7) clone 2.1 containing sequences are specifically amplified by PCR from this cloned, non specifically amplified fraction. This fraction is cloned in a T/A vector (Promega) and the inserts of these clones are

analysed by PCR with 2.1 specific primers. If 2.1 containing sequences are underrepresented in this population a further round of enrichment can be performed by repeating the amplification with vector- and 2.1 specific primers as described above. The inserts of 2.1 containing inserts are sequenced. These sequences are screened in the databases and unknown sequences (containing the previously known part) can be analysed by PCR. Clones remaining negative on negative control samples and giving positive results on the positive control samples, i.e samples of proven HepY patients, are further analysed.

The protocol described above may be repeatedwith any restriction enzyme (e.g. Bgl II or EcoR 1). In this way different cDNA-fragments arise, which leads to the elucidation of another part of the HYV genome. By alternating between these two preparations most of the HYV genome is amenable for analysis.

Detailed protocol (see also example 6 for deatails)

* Sample preparation

Total nucleic acid from 1 ml OHN serum is isolated by the Boom method, using GuSCN and silica binding.

After elution, the nucleic acid is precipitated overnight at a temperature of -20 °C, by adding 0.1 volumes of 3M NaAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample is spundown in the ultracentrifuge during one hour at 25000 rpm and a temperature of 4 °C.

After air-drying, the pellet is dissolved in a mixture of 0.2 µl RNase inhibitor and 4.8 µl DEPC-treated water.

* First strand synthesis

To the 5 µl eluate 0.7 µl of DEPC-treated water and 0.3 µl of random hexamer (50 ng/µl) is added.

This mixture is incubated during 10 minutes at 70 °C and next during 1 minute at 4 °C.

The following is added: - 1 µl of 10 X PCR buffer(200 mM Tris-HCl (pH 8.4),
500 mM KCl)

- 1 µl of a 25 mM MgCl2 solution
- 0.5 µl of a 10 mM dNTP solution

- 1 µl of 0.1 M DTT.

This mixture is incubated during 5 minutes at a temperature of 25 °C.

0.5 µl of superscript II enzyme is added.

Incubation: - 10 minutes at 25 °C.

- 50 minutes at 42 °C.

- 15 minutes at 70 °C.

After this incubation the reaction is stored on ice and followed immediately by the second-strand synthesis.

* Second-strand synthesis

The second-strand synthesis is performed according to the Boehringer Mannheim protocol.

To the 10 µl first strand reaction, the following reagents are added:

- 8 µl of 2.5 X second strand buffer

- 2 µl of DNA polymerase I (13.6 units)

- 0.5 µl of RNase H (1 unit)

Incubation: - 120 minutes at 14 °C.

- 10 minutes at 70 °C.

0.5 µl of T4 DNA polymerase (4 units) is added.

This mixture is incubated at 37 °C. for 10 minutes.

The reaction is purified by a phenol/chloroform extraction.

An overnight precipitation (-20 °C) is performed after adding 0.1 volume of 3M NaAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample is spun down in the ultracentrifuge during 1 hour at 25000 rpm and a temperature of 4 °C.

After drying, the pellet is redissolved in 15 µl of DEPC-treated water.

* BamH1 digestion

Digestion with the enzyme BamH1 is performed in a total volume of 20 µl:

- 15 µl of ds cDNA

- 4 µl of 10 X One PhorAll buffer (Promega)
- 1 µl of BamH1 (10 units)

This reaction is incubated at a temperature of 37 °C. for 15 hours.

The reaction is purified by a phenol/chloroform extraction.

An overnight precipitation (-20 °C) is performed by adding 0.1 volumes of 3M NaAc (pH 5.3) and 2.5 volumes of ice-cold 96% ethanol.

The sample is spun down in the ultracentrifuge during 1 hour at a speed of 25000 rpm and a temperature of 4 °C.

After drying, the pellet is redissolved in 1 µl of DEPC-treated water.

* Adaptor ligation

For the adaptor ligation we use the RapidLigation kit (Boehringer Mannheim)

To the 1 µl sample the following reagents are added:

- 0.8 µl adaptormix (containing 0.5 µg of 24-mer and 0.25 µg of 12-mer)
- 0.5 µl of DNA dilution buffer (5X)
- 2.5 µl of T4 DNA ligase buffer (2X)
- 0.3 µl of T4 DNA ligase

Incubation: 15 minutes at roomtemperature
1 hour at 16 °C.

* Nonspecific amplification of adaptor-ligated cDNA-molecules

For each PCR reaction the following reagents are mixed:

- 13.2 µl of DEPC-treated water
- 2 µl of 10X PCR buffer (Perkin Elmer)
- 1.6 µl of a 2.5 mM dNTP-solution
- 1 µl of the 24 mer adaptor primer (1 µg/µl)

1 µl of the ligation-product

- 0.2 µl of Perkin Elmer amplitaq

The following cycle scheme is used (in a hot-lid thermal cycler):

- 94 °C. for 30 seconds

- 30 cycles: 94 °C. 30 seconds
70 °C. 4 minutes

After performing the PCR reactions 5 µl of the PCR-product is analysed on a 1.2% agarose gel, the remainder is cleaned by the Qiaex procedure and cloned directly into the T/A vector (Promega) .

For this purpose the following ingredients were mixed:

T-vector (50 ng/µl, Promega)	1 µl
DNA ligase buffer, 10* :300mM Tris-HCl, pH 7.8 100 mM MgCl2 100 mM DTT 10 mM ATP	1 µl
insert	1 µl
T4 DNA ligase (3units/µl, Promega)	0.5 µl
bidistilled water	6.5 µl

The ligation was performed overnight at 16°C.

Semi-nested amplification of adaptor-ligated cDNA-molecules

The cloned PCR preparation was subjected to a second round of PCR

For each PCR reaction the following reagents were mixed:

- 14 µl of aqua bidest
- 2 µl of 10X PCR buffer (Perkin Elmer) (200 mM Tris-HCl (pH 8.4), 500 mM KCl, Perkin Elmer)
- 1.6 µl of a 2.5 mM dNTP-solution
- 1 µl of the Sp6 promotor or T7 promotor specific primer (1 µg/µl)
- 1 µl of the gene-specific primer (P553 or P554; 1 µg/µl)
- 1 µl of the ligation-product
- 0.2 µl of Perkin Elmer Amplitaq

The following cycle scheme was used (in a hot-lid thermal cycler):

- 94 °C. for 30 seconds
- 30 cycles: 94 °C. 30 seconds

70 °C. 4 minutes

Sequentie P553: 5-CCCGACGAACGTACGCTGAGCGTA

Sequentie P554: 5-CGCTCGATGGCGTACCACAGCTC

After performing the PCR reactions 5 µl of the PCR-product was analysed on a 1.2% agarose gel. The products were purified using the Qiagen Qiaex protocol.

The DNA then was cloned into the T/A cloning vector (Promega).

For this purpose the following ingredients were mixed:

T-vector (50 ng/µl, Promega)	1 µl
DNA ligase buffer, 10* 300mM Tris-HCl, pH 7.8 100 mM MgCl2 100 mM DTT 10 mM ATP	1 µl
insert	1 µl
T4 DNA ligase (3units/µl, Promega)	0.5 µl
bidistilled water	6.5 µl

The ligation was performed overnight at 16°C.

Electrocompetent TopF-cells (Invitrogen) were transformed with ten times diluted ligation mix with sterile water following standard procedures .

DNA of the plasmids containing 2.1 derived inserts, as deduced by PCR with SP6 and T7 primers succeeded by a PCR with 2.1 specific primers (P443 and P442), were sequenced.

Sequentie P442: 5-CACGCCCTCGACAAACAGCG

P443: 5-TCTGCACGCCTTGGTTTGCA

According to the above protocol, the sequences shown in SEQ ID NO's 17 to 26 were identified as partial clones derived from the Hepatitis Y genome.

CLAIMS

1. A method for detecting non-A-G hepatitis virus in a sample negative for HCV wherein said sample is contacted with antibodies obtainable from a cell line deposited at the ECACC under accession number 9110711 or monoclonal antibodies having the same immunoreactivity whereafter the presence of immunecomplexes formed is detected and from this the presence of non-A_G hepatitis virus is determined.

2 An immortalised human hepatocyte cell line deposited at the ECACC under accession number 98121503.

3 Use of the cell line according to claim 2 in the in vitro diagnosis of non-A-G hepatitis.

4 Hepatitis Y virus characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement.

5 Nucleic acid sequence derivable from the genome of Hepatitis Y virus or fromfragments of said genome or functional equivalents of said nucleic acid sequence, wherein Hepatitis Y virus is characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement

6 A polypeptide comprising an amino acid sequence or fragment thereof wherein said amino acid sequence is encoded by a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement, or any functional equivalents of said polypeptide.

7 Antibodies reactive with a polypeptide according to claim 3 or functional equivalents thereof.

8 Method for the detection of hepatitis Y virus in a sample comprising the steps of isolating nucleic acid from said sample and hybridising said nucleic acid with a nucleotide sequence derived from the nucleic acid sequence of claim 2.

9 Method for the detection of hepatitis Y virus in a sample according to claim 5 characterised in that said nucleotide sequence is derived from the sequence in SEQ ID NO: 1 .

10 Method for diagnosing infection with hepatitis Y virus comprising the steps of providing a sample from a patient or animal suspected of being infected with Hepatitis Y virus, providing a hepatitis Y virus according to claim 1 or a polypeptide according to claim 3 and establishing immunoreactivity of said virus or polypeptide with antibodies in said sample.

11 Method for diagnosing infection with hepatitis Y virus comprising the steps of providing a sample from a patient or animal suspected of being infected with Hepatitis Y virus, providing an antibody according to claim 4 and establishing whether immunoreactive components are present in said sample.

12 Method for growing Hepatitis Y virus (HYV) comprising providing cells infected with HYV and propagating said cells in vitro, wherein HYV is characterised in that it comprises a genome comprising a nucleotide sequence hybridisable to the sequence of SEQ ID NO: 1 or its complement or the sequence of SEQ ID NO: 2 or its complement.

13 A vaccine composition comprising a polypeptide according to claim 3 in substantially isolated form mixed with a pharmaceutically acceptable excipient

14 A vaccine composition comprising a nucleic acid sequence according to claim 2 in substantially isolated form mixed with a pharmaceutically acceptable excipient

Figures 1/6

Figure 1

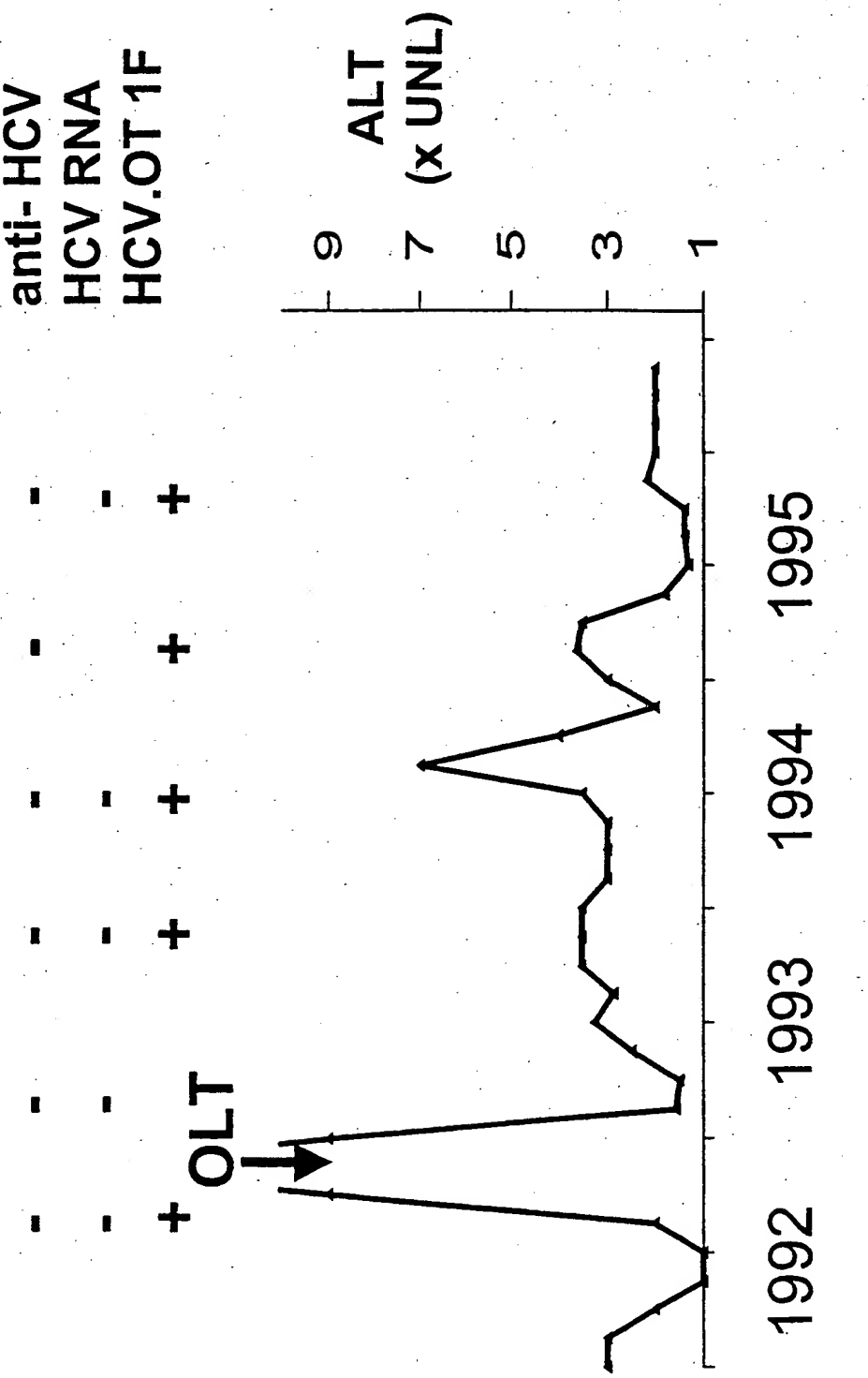

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Figure 2

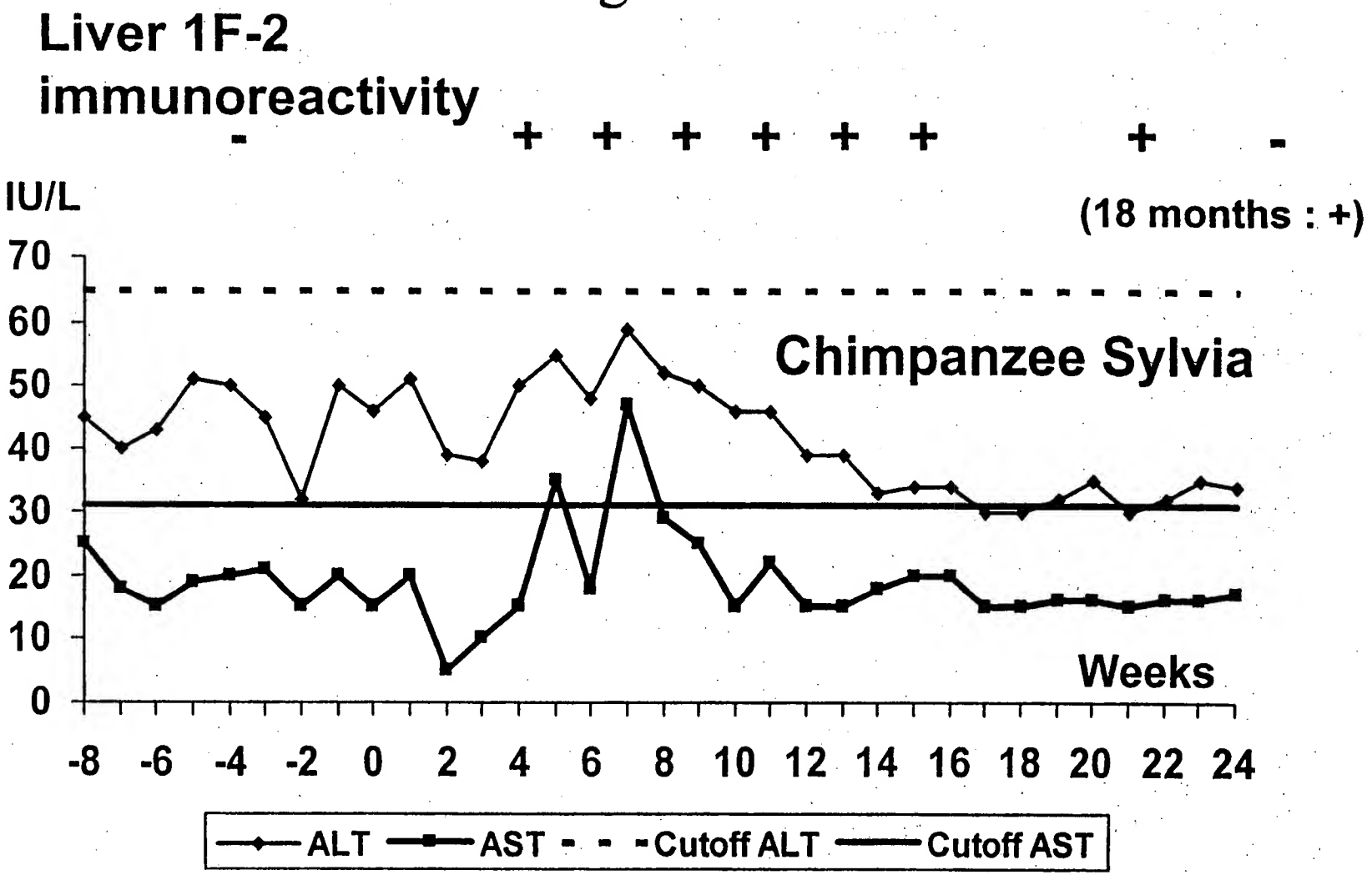

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Figure 3

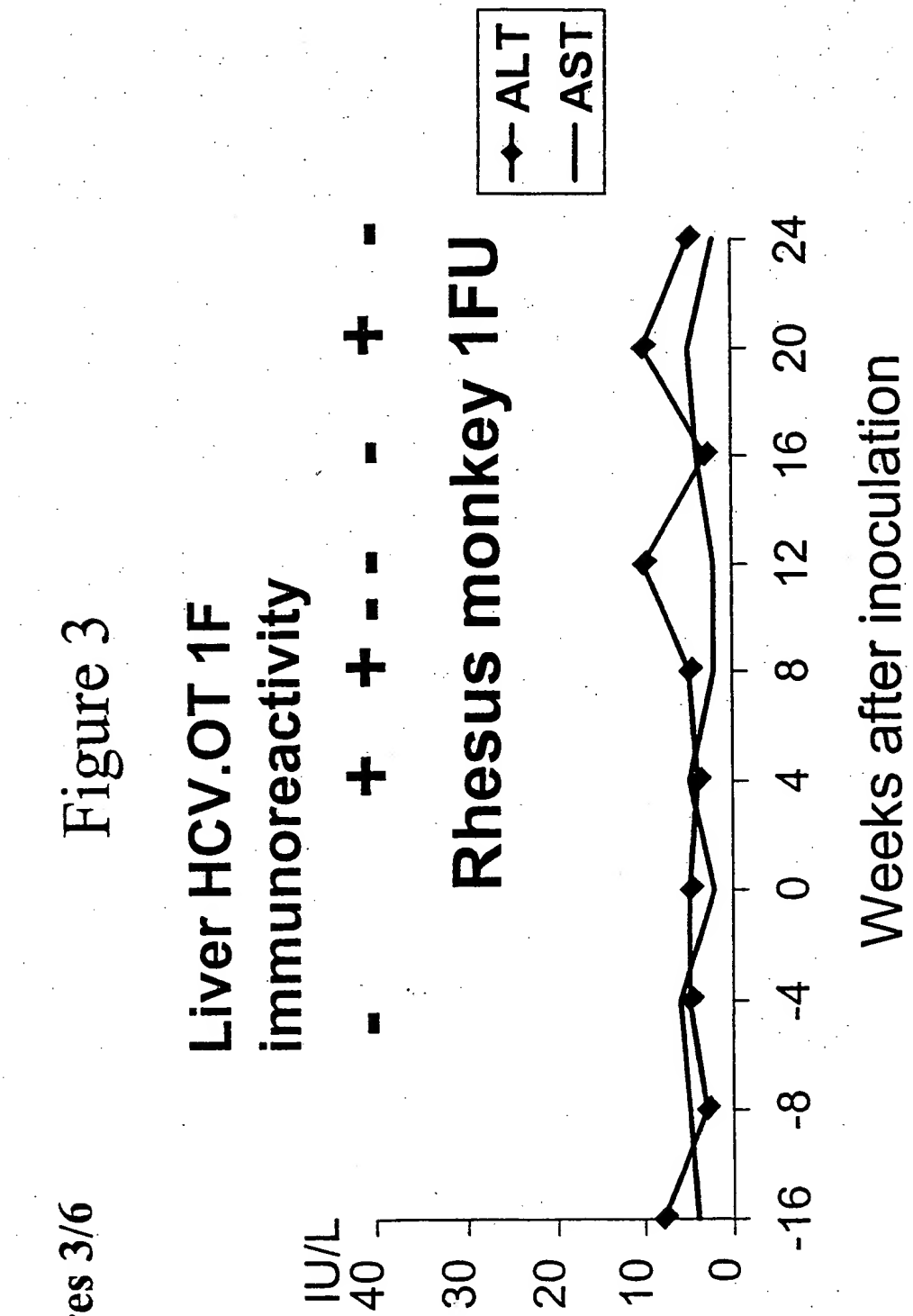

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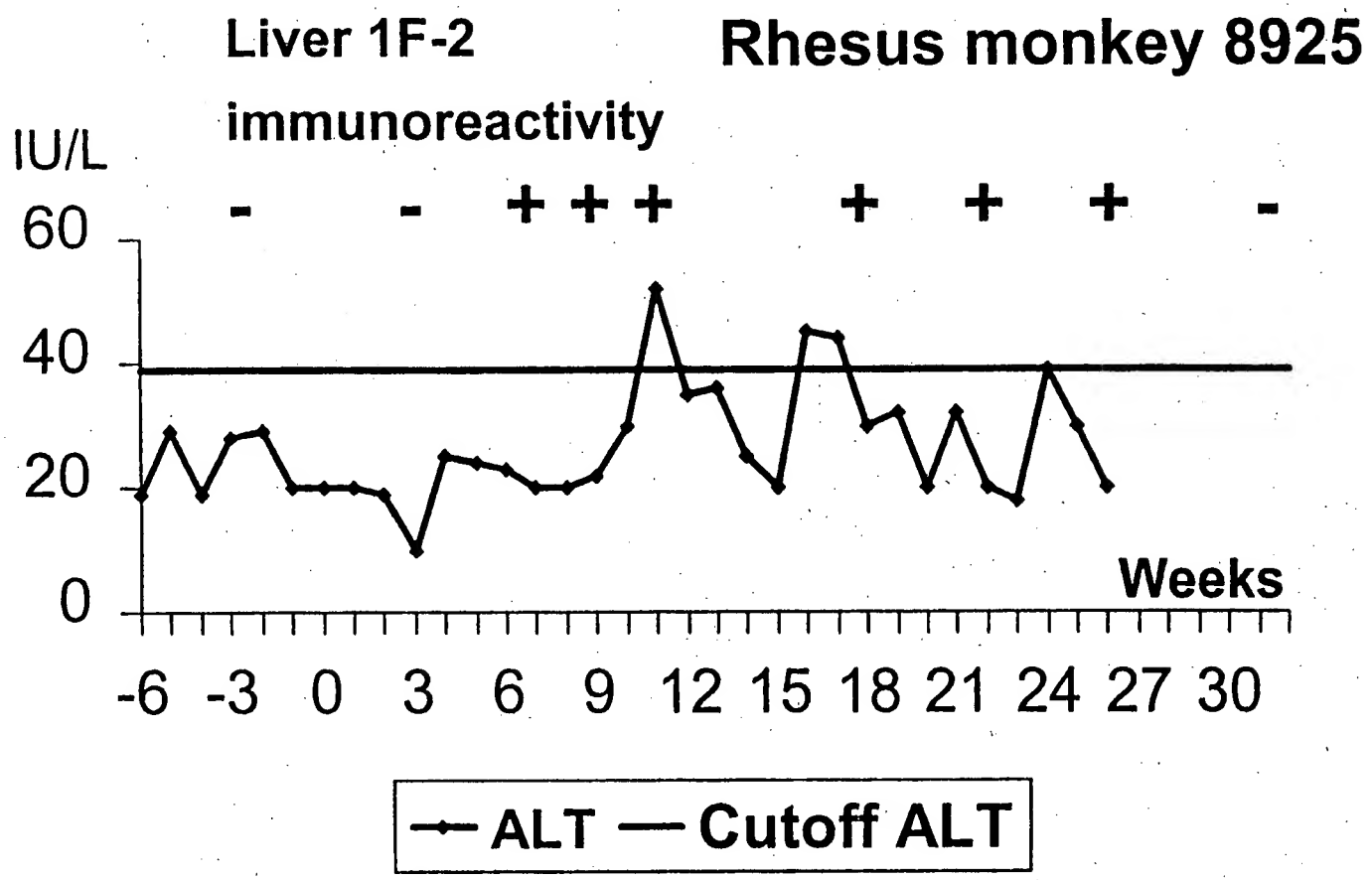
Figure 4



Figures 5/6

Figure 5

Cesium chloride gradient Plasma of patient OHN

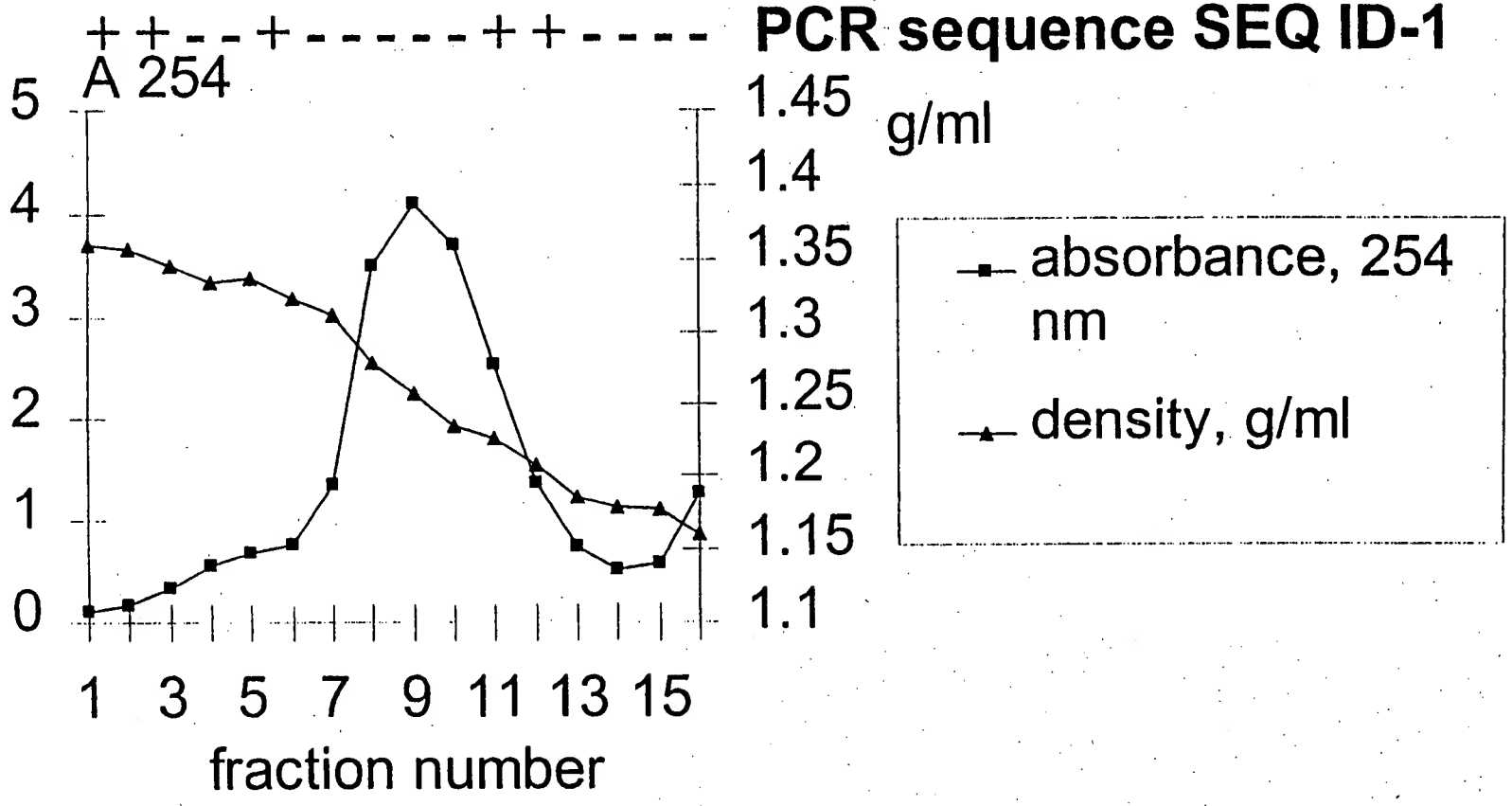


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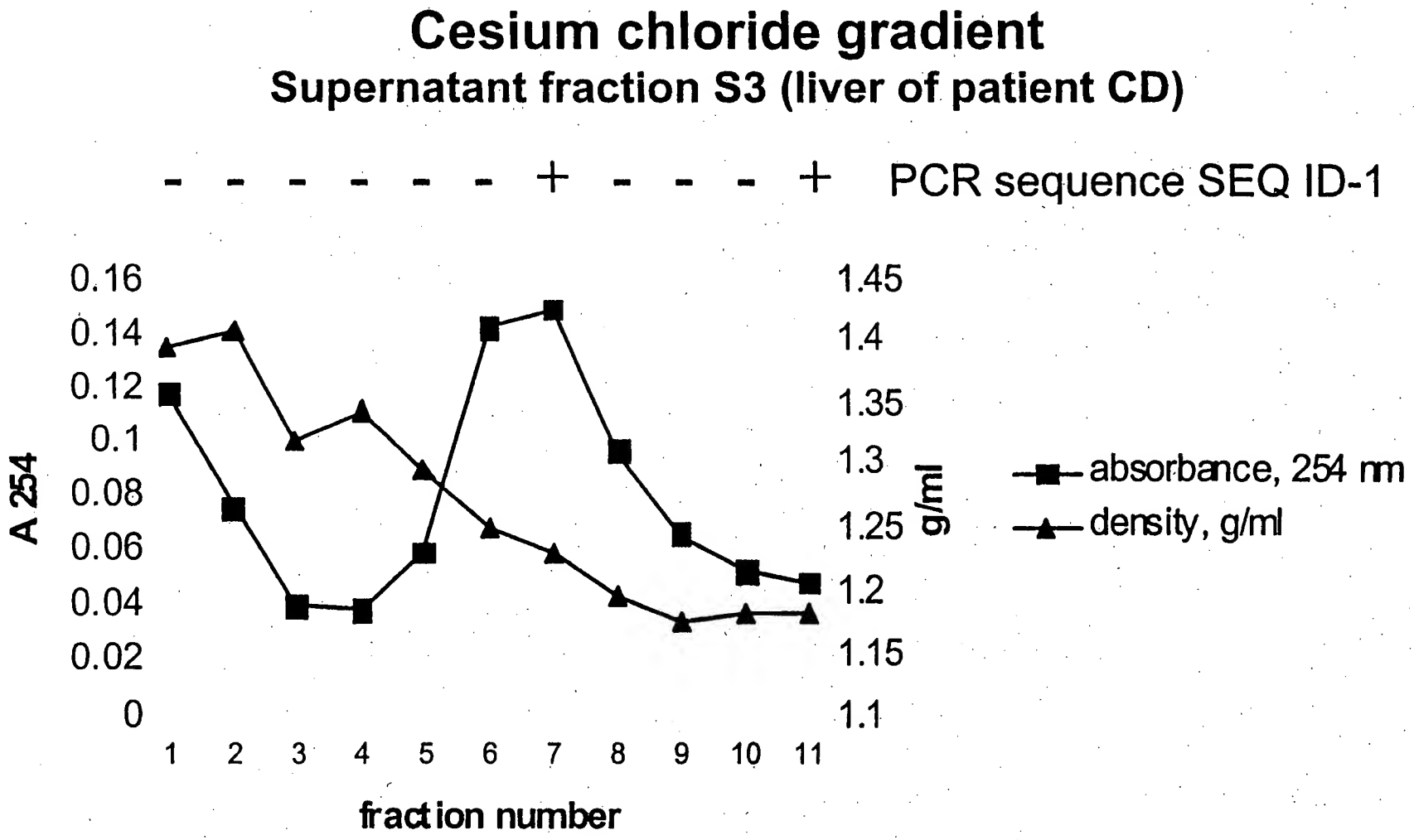
Figure 6



SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Akzo Nobel N.V.
(B) STREET: Velperweg 76
(C) CITY: Arnhem
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 6824 BM
(G) TELEPHONE: 0412 666379
(H) TELEFAX: 0412 650592

(ii) TITLE OF INVENTION: Hepatitis Y Virus

(iii) NUMBER OF SEQUENCES: 26

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 304 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GATCACAAGC AACTGCCCGA CGAACGTACG CTGAGCGTAT TCGTCGACGA ACTGCACGCC 60

CTCGACAAAC AGCGCCTGTC CGGCAAGCTG TCCGAGGAGT TCAACCGCGC CTATACCGGC 120

ATGTCCAGCG TGGCCAAAGC CACTGCCCGG CGCGTTGGCC GACTGGACGC CCAGGCGCTG 180

CAAAGCCAAG GCGTGCAGAC GCTGCTCGAG GCCCACCGCA ACTGGAGCAA GCCCGAGCTG 240

TGGTACGCCA TCGAGCGCGC CGGCAAGGTT TACACCTACG ATTACTACCT GACCGCACTG 300

GATC 304

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GATCGABGTG CAACACGCCC GCCTATHACG GCGCGTATTG CTTGTBGCAG CCTGAGTGCA 60

GCATTAGATT AGCCAATTAT CTGGGGCACC ATCATAAGCA GAAGGGATAA GCATGGCGCT 120

CACCGACCAA TCCACCCGCA CCCGTACCGG CGAAGAACTC GACGCTGCCG TCATCGACGC 180

CTATCTCAAG GCCCATATTC CCGGCCTGAG TGGCGAGGCC GG 222

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGTACGCTGA GCGTA 15

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGCGTACCAC AGCTC 15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CACGCCCTCG ACAAACAGCG 20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCTGCACGCC TTGGTTTGCA 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTGCAACACG CCCGC 15

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCGGCCTCGC CACTC 15

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACGGCGCGTA TTGCTTGT 18

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCCGGGAATA TGGGCCTT 18

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ser Gln Ala Thr Ala Arg Arg Thr Tyr Ala Glu Arg Ile Arg Arg Arg
1 5 10 15

Thr Ala Arg Pro Arg Gln Thr Ala Pro Val Arg Gln Ala Val Arg Gly
20 25 30

Val Gln Pro Arg Leu Tyr Arg His Val Gln Arg Gly Gln Ser His Cys
35 40 45

Pro Ala Arg Trp Pro Thr Gly Arg Pro Gly Ala Ala Lys Pro Arg Arg
50 55 60

Ala Asp Ala Ala Arg Gly Pro Pro Gln Leu Glu Gln Ala Arg Ala Val
65 70 75 80

Val Arg His Arg Ala Arg Arg Gln Gly Leu His Leu Arg Leu Leu Pro
85 90 95

Asp Arg Thr Gly
100

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ile Thr Ser Asn Cys Pro Thr Asn Val Arg Xaa Ala Tyr Ser Ser Thr
1 5 10 15

Asn Cys Thr Pro Ser Thr Asn Ser Ala Cys Pro Ala Ser Cys Pro Arg
20 25 30

Ser Ser Thr Ala Pro Ile Pro Ala Cys Pro Ala Trp Pro Lys Pro Leu
35 40 45

Pro Gly Ala Leu Ala Asp Trp Thr Pro Arg Arg Cys Lys Ala Lys Ala
50 55 60

Cys Arg Arg Cys Ser Arg Pro Thr Ala Thr Gly Ala Ser Pro Ser Cys
65 70 75 80

Gly Thr Pro Ser Ser Ala Pro Ala Arg Phe Thr Pro Thr Ile Thr Thr
85 90 95

Xaa Pro His Trp Ile
100

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 101 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Asp His Lys Gln Leu Pro Asp Glu Arg Thr Leu Ser Val Phe Val Asp
1 5 10 15

Glu Leu His Ala Leu Asp Lys Gln Arg Leu Ser Gly Lys Leu Ser Glu
20 25 30

Glu Phe Asn Arg Ala Tyr Thr Gly Met Ser Ser Val Ala Lys Ala Thr
35 40 45

Ala Arg Arg Val Gly Arg Leu Asp Ala Gln Ala Leu Gln Ser Gln Gly
50 55 60

Val Gln Thr Leu Leu Glu Ala His Arg Asn Trp Ser Lys Pro Glu Leu
65 70 75 80

Trp Tyr Ala Ile Glu Arg Ala Gly Lys Val Tyr Thr Tyr Asp Tyr Tyr
85 90 95

Leu Thr Ala Leu Asp
100

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Asp Pro Val Arg Ser Gly Ser Asn Arg Arg Cys Lys Pro Cys Arg Arg
1 5 10 15

Ala Arg Trp Arg Thr Thr Ala Arg Ala Cys Ser Ser Cys Gly Gly Pro
20 25 30

Arg Ala Ala Ser Ala Arg Leu Gly Phe Ala Ala Pro Gly Arg Pro Val
35 40 45

Gly Gln Arg Ala Gly Gln Trp Leu Trp Pro Arg Trp Thr Cys Arg Tyr
50 55 60

Arg Arg Gly Xaa Thr Pro Arg Thr Ala Cys Arg Thr Gly Ala Val Cys
65 70 75 80

Arg Gly Arg Ala Val Arg Arg Arg Ile Arg Ser Ala Tyr Val Arg Arg
85 90 95

Ala Val Ala Cys Asp
100

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Ile Gln Cys Gly Gln Val Val Ile Val Gly Val Asn Leu Ala Gly Ala
1 5 10 15

Leu Asp Gly Val Pro Gln Leu Gly Leu Ala Pro Val Ala Val Gly Leu
20 25 30

Glu Gln Arg Leu His Ala Leu Ala Leu Gln Arg Leu Gly Val Gln Ser
35 40 45

Ala Asn Ala Pro Gly Ser Gly Phe Gly His Ala Gly His Ala Gly Ile
50 55 60

Gly Ala Val Glu Leu Leu Gly Gln Leu Ala Gly Gln Ala Leu Phe Val
65 70 75 80

Glu Gly Val Gln Phe Val Asp Glu Tyr Ala Gln Arg Thr Phe Val Gly
85 90 95

Gln Leu Leu Val Ile
100

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Ser Ser Ala Val Arg Xaa Xaa Ser Xaa Val Xaa Thr Leu Pro Ala Arg
1 5 10 15

Ser Met Ala Tyr His Ser Ser Gly Leu Leu Gln Leu Arg Trp Ala Ser
20 25 30

Ser Ser Val Cys Thr Pro Trp Leu Cys Ser Ala Trp Ala Ser Ser Arg
35 40 45

Pro Thr Arg Arg Ala Val Ala Leu Ala Thr Leu Asp Met Pro Val Xaa
50 55 60

Ala Arg Leu Asn Ser Ser Asp Ser Leu Pro Asp Arg Arg Cys Leu Ser
65 70 75 80

Arg Ala Cys Ser Ser Ser Thr Asn Thr Leu Ser Val Arg Ser Ser Gly

85 90 95

Ser Cys Leu Xaa
100

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 376 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CGCCCGGGCA GGTCTGTTCA TCCTCGTCAC CTTCGTCGCC CCGATCGGTA CCATGCTGTG 60

GCGCACGTGC ATCACCCGAC CGTGGCCGAG CTGATTCCCC TGACCCTGGC CGAGCTCGAA 120

CGCTGGGATG ATCACAAGCA ACTGCCGGAC GAAAAAACCC TGCAGGTCTT CGCCAGCGAA 180

CTGCATGGCC TTAACCAGCA GCGCCTGTCC GGCAAGCTCT CCGAAGAACT CAACCGCGCC 240

TATACCGGCA TGTCCAGCGT GGTCAAAGCC ACTGCCCGGC GCGTTGGCCG ACTGGACGCC 300

CAGGCGCTGC AAACCAAGGC GTGCGGACGC TGCTCGAGGC CCACCGCAAC TGGAGCAAGC 360

CCGAGCTGTG GTACGC 376

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 378 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CGCCCGGGCA GGTCTGTTCA TCCTCGTCAC CTTCGTCGCC CCGATCGGTA CCATGCTGTG 60

GCGCAGCGTG CATCACCCGA CCGTGGCCGA GCTGATTCCC CTGACCCTGG CCGAGCTCGA 120

ACGCTGGGAT GATCACAAGC AACTGCCGGA CGAAAAAACC CTGCAGGTCT TCGCCAGCGA 180

ACTGCATGGC CTTAACCAGC AGCGCCTGTC CGGCAAGCTC TCCGAAGAAC TCAACCGCGC 240

CTATACCGGC ATGTCCAGCG TGGTCAAAGC CACTGCCCGG CGCGTTGGCC GACTGGACGC 300

CCAGGCGCTG CAAAGCCAAG GCGTGCGGAC GCTGCTCGAG GCCCACCGCA ACTGGAGCAA 360

GCCCGAGCTG TGGTACGC 378

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 404 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AAGAAGCGCG TAGCCCCATT TGTGTTCGTG GTGCCAATGC TGCTGTTCAT CCTCGTCACC 60

TTCGTCGCCC CGATCGGTAG GATCCTGTGG CGCAGCGTGC ATCACCCGAC CGTGGCCGAG 120

CTGATTCCCG TGACCCTGGC CGAGTCGAAC GCTGGGATGA TCACAAGCAA CTGCCGGACG 180

AAAAAACCCT GCAGGTCTTC GCCAGCGAAC TACNTNCCTT AACCAGCAGC GCCTGTCCGG 240

CAACTCTCCG AAGAACTCAA CCACGCCTAT ACCGGCATAT CCTGCGTGCT CAAATTTACT 300

GCCCGGCGCG TTGGCCGACT GGACGCCCAG GCGCTGCAAA GCCAAGGCGT GCAGACGCTG 360

CTCGAGGCCC ACCGCAACTG GAGCAAGCCC GAGCTGTGGT ACGC 404

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 366 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: unsure
(B) LOCATION:363..366

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CGCTGAGCGT ATTCGTCGAC GAACTGCACG CCCTCGACAA ACAGCGCCTG TCCGGCAAGC 60

TGTCCGAGGA GTTCAACCGC GCCTATACCG GCATGTCCAG CGTGGTCAAA GCCACTGCCC 120

GGCGCGTTGG CCGACTGGAC GCCCAGGCGC TGCAAAGCCA AGGCGTGCAG ACGCTGCTCG 180

AGGCCCACCG CAACTGGAGC AAGCCCGAGC TGTGGTACGC CATCGAGCGC GCCGGCAAGG 240

TTTACACCTA CGATTACTAC CTGACCGGAC TGCATCTCGA GATCTATACT GACTAATCCC 300

TTGACCATCG CGACCAGAAG CATATCACCT CCCCGCCAAT TCGCAATATA CCTATANTTA 360

TANNNN 366

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CACTTGTGGG AGGGGGGTTG GGGGAGAGGG TGAATCTGTC ACCATCTCCC CAGCCCTCTC 60

CGTAAACGGG AGAGGGAGCT ACTGATCGAC ACTTGCTGTG CGCCCTGTTT TACGGAGTAA 120

CACCTTGTCA TCCCTGACCA CCAGCGAAGC CGGCCAAGCC GCCAGCGCCC GTCGCAAGAA 180

GTGCGTCGCC GCCTTTCTGT TCGTGGTGCC ACTGCTGCTG TTCATCCTCG TCACCTTCGT 240

CGCCCCGATC GGTACCATGC TGTGGCGCAG CGTGCATCAC CCGACCGTGG CCGAGCTGAT 300

TCCCCTGACC CTGGCCGAGC TCGAACGCTG GGATGATCAC AAGCAACTGC CGGACGAAAA 360

AACCCTGCAG GTCTTCGCCA GCGAACTGCA TGGCCTTAAC CAGCAGCGCC TGTCCGGCAA 420

GCTCTCCGAA GAACTCAACC GCGCCTATAC CGGCATGTCC AGCGTGGTCA AAGCCACTGC 480

CCGGCGCGTT GGCCGACTGG ACGCCCAGGC GCTGCAAAGC CAAGGCGTGC AGACGCTGCT 540

CGAGGNNCAC CGCAACTGGA GCAAGCCCGA GCTGTGGTAC GC 582

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 513 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CCAATATGTT GGAGCTGCTT TGATATGTTC GACATATAGT CGACCTGAAT ACTGATGAAT 60

TAGTATTTGG TTAGTGTGGC GGTCGGGGCA GGTCCAGCGC AGGTCGCAAG AAACGCCTCG 120

CTGCCTTTCT CTTCGTGGTA CCGCTGCTGC TGTTCATCAT CGTCACCTTC GTCGCCCCGA 180

TCGGTACCAT GCTGTGGCGC AGCGTGCATC ACCCGACCGT GGCCGAACTG ATTCCTCTGA 240

CCCTGGCCGA ACTCGAGCGC TGGGACGATC ACAAGCAACT GCCCGACGAA CGTACGCTGA 300

GCGTATTCGT CGACGAACTG CACGCCCTCG ACAAACAGCG CCTGTCCGGC AAGCTGTCCG 360

AGGAGTTCAA CCGCGCCTAT ACCGGCATGT CCAGCGTGGT CAAAGCCACT GCCCGGCGCG 420

TTGGCCGACT GGACGCCCAG GCGCTGCAAA GCCAAGGCGT GCAGACGCTG CTCGAGGCCC 480

ACCGCAACTG GAGCAAGCCC GAGCTGTGGT ACG 513

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGCTGAGCGT ATTCGTCGAC GAACTGCACG CCCTCGGCAA ACAGCGCCTG TCCGGCAAGC 60

TGTCCGAGGA GTTCAACCGC GCCTATACCG GCATGTCCAG CGTGGTCAAA GCCACTGCCC 120

GGCGCGTTGG CCGACTGGAC GCCCAGGCGC TGCAAAGCCA AGGCGTGCAG ACGCTGCTCG 180

AGGCCCACCG CAACTGGAGC AAGCCCGAGC TGTGGTACGC CATCGAGCGC GCCGGCAAGG 240

TTTACACCTA CGATTACTAC CTGACCGCAC TGGATCTGGA GATGCACCCC GACGAGGGCA 300

TCCAGGTGCG CCAGGACACG CAGATCTATC TGCAGCTGTA TTCCAAGACC CTGAACATGG 360

CGCTGGTCAT CACCCTGCTC TGCGCCCTGC TCGGCTACCC GGTGGCCTAC TACCT 415

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 530 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGCTGAGCGT ATTCGTCGAC GAACTGCACG CCCTCGACAA ACAGCGCCTG TCCGGCAAGC 60

TGTCCGAGGA GTTCAACCGC GCCTATACCG GCATGTCCAG CGTGGTCAAA GCCACTGCCC 120

GGCGCGTTGG CCGACTGGAC GCCCAGGCGC TGCAAAGCCA AGGCGTGCAG ACGCTGCTCG 180

AGGCCCACCG CAACTGGAGC AAGCCCGAGC TGTGGTACGC CATCGAGCGC GCCGGCAAGG 240

TTTACACCTA CGATTACTAC CTGACCGCAC TGGATCTGGA GATGCACCCC GACGAGGGCA 300

TCCAGACCTG CCCGGGCGGC CGCTCGACCC CTATAGTGAG TAATCCCGCG GCCATGGCGG 360

CCGGGAGCAT GCGACGTCGG GCCCAATACG CCCTATAGTG AGTCGTATTA AAATTCACTG 420

GCCGTCGTTT TACAANGTNG TGAATGGNAA ANCCTGGCGT TACCCAACTT AATCGCCTTG 480

CAGCACATCC CCCTTTCGCC AGCTGGCGTA ATAGCGAAGA GGCCCGCACC 530

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 319 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CGCTGAGCGT ATTCGTCGAC GAACTGCACG CCGTCGACAA ACAGCGCCTG TCCGGCAAGC 60

TGTCCGAGGA GTTGAACCGC GCGTATACCG GCATGTCCAG CGTGGTCAAA GCCAGTGCCC 120

GGCGCGTTGG CCGAGTGGAC GCCCAGGCGC TGCAAAGCCA AGGCGTGCAG AGCGCTGCTC 180

GAGGCCCACC GCAACTGGAG CAAGCCCGAG CTGTGGTACG CCATCGAGCG CGCCGGCAAG 240

GTTTACACCT AGGATTACTA CCTGACCGCA CTGGATCTGG AGATGCACCC CGACGAGGGC 300

ATCCAGACCT GCCCGGGCG 319

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 368 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GATCACAAGC AACTGCCCGA CGAACGTACG CTGAGCGTAT TCGTCGACGA ACTGCACGCC 60

CTCGACAAAC AGCGCCTGTC CGGCAAGCTG TCCGAGGAGT TCAACCGCGC CTATACCGGC 120

ATGTCCAGCG TGGCCAAAGC CACTGCCCGG CGCGTTGGCC GACTGGACGC CCAGGCGCTG 180

CAAAGCCAAG GCGTGCAGAC GCTGCTCGAG GCCCACCGCA ACTGGAGCAA GCCCGAGCTG 240

TGGTACGCCA TCGAGCGCGC CGGCAAGGTT TACACCTACG ATTACTACCT GACCGCACTG 300

GATCTGGAGA TGCACCCCGA CGAGGGCATC CAGGCGCGCC AGGACACGCA GATCTACCTG 360

CCCGGGCG 368